Seeing the light: Combining a behavioural and genetic approach to unravel the mysteries of the larval visual system of *Drosophila melanogaster*

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Abstract

Using a behavioural and genetic approach, we employed single-gene mutations and photoreceptor cell ablations to study the molecules and cell-types underlying larval response to light *Drosophila* larvae modulate their locomotion in response to light In the Checker Assay the response to light is defined as an increased residence time in dark quadrants versus lit. In the ON/OFF Assay, it is in part defined as a decreased path length in the light. Previously, mutations in genes, which function in the adult phototransduction cascade were found to abolish light perception as defined by a reduction in path length. This response to light was reported to be mediated by rhodopsins, other than Rh1, via a pathway similar to the one present in the adult visual system. After undertaking a similar genetic approach in the Checker Assay, the response to light measured in this assay appears also to be mediated through a similar pathway. Mutations in *sine oculis (so),* a homeobox gene necessary for proper visual system development, and targeted expression of the cell death gene *head involution defective (hid),* to larval photoreceptor neurons, abolished light response as measured in the Checker Assay. Thus, mutations affecting development of larval visual system suggest that this response to light is also housed in the larva's main photoreceptor organ, the BO.

The modular GAL4 system was used to target expression of cell death genes, *rpr* and *hid,* to Rh5 and Rh6 expressing larval photoreceptor cells. In strains tested in the ON/OFF Assay, in which Rh5 cells are missing, the response to light is abolished, as

measured by both decreased path length and increased head swinging behaviour in the light. In a strain in which Rh6 photoreceptor cells are ablated, this response to light is not abolished. This suggests that Rh5 mediates responses in the ON/OFF Assay, which were previously abolished by mutations in genes operating in the adult phototransduction cascade. Thus Rh5, not Rh6, appears to be necessary in mediating the response to light carried out via a pathway similar to the operating in adult phototransduction. In both ablated strains, Rh5-gal4xUAS-*rpr* and Rh5-gal4xUAS-*hid*, the integrity of remaining photoreceptor cells is not compromised, and in the latter strain, the extent of ablation appears to be complete.

Previously in the ON/OFF assay, mutations and ablations of cell-types were found to disrupt only a subset of behaviours associated with the larval perception of light. Based upon this evidence it was surmised that Rh1 mediates a basic independent visual system, which operates in the larva. However, uncovering the possible roles in this system was hindered, as parental control strains did not respond.

Chapter I: Introduction

A Systematic Genetic Approach has been Deployed to Understand a Plethora of Behavioural Responses

Seymour Benzer first pioneered a genetic approach in the dissection of behaviour with his initial experiment (Benzer *et al.*, 1967), which entailed the isolation of phototactic *Drosophila* mutants. Benzer proposed and implemented a strategy through which changes in defined behaviour could be correlated with single-gene mutations (Benzer *et al.*, 1967; Hotta and Benzer, 1970). This simple paradigm has been extended and deployed to untangle a wide variety of behavioural responses. This systematic genetic approach, which found its beginning in the model organism, *Drosophila melanogaster,* is still used to study other aspects of its behaviour and has also been applied to other model systems such as the nematode, *C. Elegans*, the honey bee, *Apis mellifera*, and more recently, *Mus Murus* (Wen *et al.*, 1997; Vitaterna *et al.*, 1994). The simple nervous system found in *C. Elegans*, along with genetic tools have permitted the identification of novel genes, which function in learning and memory (Wen *et al.*, 1997; Morrison *et al.*, 1999). The honey bee has also served as an excellent model for the study of learning and memory in

that foraging trips to flowers require the bee to learn celestial and seasonal cues (reviewed by Menzel and Muller, 1996). In *Drosophila*, complex behaviours such as learning and memory (Aceves-Pina *et al.*, 1979; Quinn *et al.*, 1967; Tully *et al.*, 1994), courtship (reviewed by Hall *et al.*, 1994; Hall *et al.*, 1998) and circadian rhythm behaviours (reviewed by Dunlap *et al.*, 1999; Renn *et al.*, 1999) have been extensively studied. Implicit in the performance of such behaviours are the sensory modalities, vision (Benzer *et al.*, 1967; Hotta and Benzer, 1970; Heisenberg *et al.*, 1977; Stark *et al.*, 1980; Miller *et al.*, 1981; Godoy-Herrera *et al.*, 1984; Sawin-McCormack *et al.*, 1995; Busto *et al.*, 1999; Iyengar *et al.*, 2000; Hassan *et al.*, 2000), olfaction (Anholt *et al.*, 1996; Lilly *et al.*, 1989) and auditory response (Eberl *et al.*, 1997).

Intrinsic and Extrinsic Features, which make Drosophila melanogaster an Attractive Model

Drosophila melanogaster is an attractive model for queries into the biology underlying complex behaviours, while genetics continues to be a fundamental tool in facilitating our understanding. Some 50 years after the father of genetics, Gregor Mendel conducted his breeding experiments in a monastery garden, Thomas Hunt Morgan selected *Drosophila* melanogaster for his study of cytogenetics (Hickman *et al.*, 1998). This marked the dawn of *Drosophila* research. Morgan had selected an organism, which conferred several obvious

advantages. Initially, the presence of polytene chromosomes in *Drosophila's* salivary glands permitted the map positions of genes to be correlated with physical features of the chromosome (Ashburner, 1989). Furthermore, their presence facilitated the identification of the breakpoint locations of chromosome rearrangements (Greenspan, 1997). Since then, the availability of unique genetic and molecular techniques has helped realize the potential for isolation and analysis of mutations. These techniques include the use of unique tools such as balancer chromosomes. Another technique, which has had profound implications for behavioural analyses, was the production of composite individuals or mosaics by genetic methods. Other intrinsic features which makes *Drosophila* an attractive model, include low maintenance, a fast generation time of 10-11 days at 25°C and high fecundity since females may lay anywhere from 70 to 3000 eggs over their lifespans (Ashburner, 1989).

Drosophila's life cycle is divided into three main stages: embryogenesis, larval and metamorphosis. Embryogenesis begins as early as 2.5 hours after fertilization (AF) and 22 hours (AF) the first instar free-living larvae hatch from the fertilized egg (Browder, 1980). The larva subsequently undergoes two larval molts, passing through the second and third instar larval stages in the process (Roberts, 1986). Pupation occurs at 5 days (AF) and is followed

by metamorphosis as the larva develops into an adult. The adult fly emerges from the pupal case 9-10 days after fertilization (Roberts, 1986).

Drosophila melanogaster has Two Distinct Visual Systems: a Rudimentary Larval Visual System and a More Complex Adult Visual System

Of the two visual systems present in *Drosophila melanogaster*, larval and adult, much attention has been devoted to the latter. Benzer's behavioural approach along with Pak's isolation of phototransduction mutants through the identification of ERG (ElectroRetinoGram)-defective mutants proved prosperous in the adult visual system research (Benzer *et al.*, 1967; Hotta and Benzer, 1970; Pak *et al.*, 1979). The picture in the larva, from a genetic and molecular standpoint is only just emerging. The larval visual system will be considered from a morphological and developmental standpoint before delving into strategies to untangle the network of genes, molecules and cell-types underlying larval visual system function.

The larval visual system of *Drosophila* is morphologically quite simple, consisting of two bilaterally symmetrical organs, each housing 12 photoreceptor cells. These organs, termed Bolwig's organs (BO) are named after N. Bolwig who first described the main photoreceptive organs in the

house fly, *Musca domestica* (Bolwig, 1946). The photoreceptor cells extend their axons fasciculating into the Bolwig's nerve (BN), which projects ventrally and then posteriorly finding its target in the central brain (Steller *et al.*, 1987; Schmucker *et al.*, 1992; Green *et al.*, 1993; Campos *et al.*, 1995; Schmucker *et al.*, 1997). This simple system begins to develop in embryogenesis from BO and the optic lobe anlagen (OLA) precursor cells situated in the posterior dorso lateral region of the embryonic head (Green *et al.*, 1993; Campos *et al.*, 1995; Namba *et al.*, 1999).

Initially, the BO and OLA precursor cells are not discernible at a morphological level, however the precursor cells can be differentiated on the basis of cell-specific molecular markers (Schmucker *et al.*, 1997). Both groups of precursor cells, express *sine oculis* (*so*), which is necessary for the proper development of larval and adult visual system (Cheyette *et al.*, 1994, Serikaku and O'Tousa, 1994). The *so* gene is expressed prior to other genes required in visual system development (Serikaku and O'Tousa, 1994). The precursor cells are distinguishable in that BO, or photoreceptor cells express the transcription factor Kruppel (*kr*), whereas the OLA precursor cells express the *disconnected* (*disco*) gene (Schmucker *et al.*, 1997; Campos *et al.*,1995; Lee *et al.*, 1991). As embryogenesis proceeds, the precursor cells begin to differentiate and the optic lobe invaginates and the OLA and BO separate and the BO, now forms a distinct cell cluster (Schmucker *et al.*,

1997). As the BN finds its target in the brain, three transcription factors are implicated in the development of this precise projection, *kr*, *glass* (*gl*) and *disco* (Schmucker *et al.*, 1997). As mentioned, *kr* and *gl* are expressed in the photoreceptor cells, the latter is necessary for proper photoreceptor differentiation (Moses *et al.*, 1989; Schmucker *et al.*, 1992; Campos *et al.*, 1995).

The adult visual system is morphologically more complex than the larval visual system. It consists of the compound eyes and the simple eyes, also known as ocelli. The compound eyes are comprised of roughly 750 ommatidia, found in a hexagonal array on the eye surface. Each individual ommatidium is comprised of 20 cells, 8 of which are photoreceptor cells (R cells) (reviewed by Smith *et al.*, 1991). The neurons of photoreceptor cells project in a stereotypic pattern to the optic lobes with R1-R6 axons synapsing in the first optic ganglia, the lamina and R7 and R8 synapsing in the second optic ganglia, the medulla. The photoreceptor cells, contain rhodopsin, a visual pigment located in the special light receptor organ, the rhabdomere. These R cells in turn are classified into three types R1-R6, R7 and R8, based on location of rhabdomere, opsin spectral sensitivity and projection patterns (reviewed by Smith *et al.*, 1991). The outer photoreceptor cells R1-R6, express the major blue-absorbing opsin Rh1, encoded by the *ninaE* gene (O'Tousa *et al.*, 1985). The inner photoreceptor cells R7 and R8 express

rhodopsins, Rh3/Rh4 (Zuker *et al.*, 1987; Montell *et al.*, 1987) and Rh5/Rh6, respectively, in non-overlapping subsets of cells (Papatsenko *et al.*, 1997; Huber *et al.*, 1996). Both Rh3 and Rh4 are UV-absorbing pigments (Feiler *et al.*, 1992) and Rh5 and Rh6 are blue and green-absorbing, respectively (Salcedo *et al.*, 1999). Interestingly, the location of R7 cells expressing Rh3 are always found above R8 cells, which express Rh5 and this pattern is analogous for Rh4 and Rh6-expressing cells (Papatsenko *et al.*, 1997; Chou *et al.*, 1996;1999).

Rhodopsin is comprised of two basic parts: an opsin moiety and a chromophore. Structurally, the opsin moiety is comprised of seven transmembrane segments and is covalently attached to a chromophore at a lysine residue in the seventh transmembrane domain. Upon absorption of light rhodopsin is activated to metarhodopsin, which leads to a conformational change in the protein, thus initiating the adult phototransduction cascade (reviewed by Montell *et al.*, 1999). The conformational change in rhodopsin activates a heterotrimeric G-protein, which in turn activates the enzyme Phospholipase C (PLC), encoded by *norpA* (Bloomquist *et al.*, 1988). PLC, in turn, catalyzes the breakdown of phosphotidyl-inositol bisphosphate (PIP_2) into two intracellular messengers: Inositol trisphosphate (IP_3) and Diacylglycerol (DAG). These secondary messengers are believed to mediate calcium influx through the opening of

calcium channels (encoded by *trp* and *trpl*) (Hardie *et al.*, 1992; Montell *et al.*, 1989; Scott *et al.*, 1997; Chyb *et al.*, 1999), which ultimately leads to the depolarization of the photoreceptor cell (Fig. I-1.). The mechanism whereby the final step occurs is presently under contention (reviewed by Montell *et al.*, 1999).

Predominant Genetic Techniques Employed in Behavioural Analyses: Mutational Analyses and Ectopic Expression of Genes

The genetic strategies successful in defining the adult visual system have been applied in unraveling the mysteries of the larval visual system. Central again to understanding this system has been mutational analysis, a reoccurring theme in *Drosophila* biology. Since the outset, mosaic analyses have been particularly informative, (Benzer *et al.*, 1969; Hotta and Benzer, 1970; Meyerowitz and Kankel, 1978) especially in adult behaviour studies. Sex mosaics or gynandromorphs, which have genotypically male and female cells, have assisted in determining the focus of a behavioural mutation. In such a manner, regions of the brain specifying sex-specific behaviours were first identified as well as the isolation of mutations for phototactic behaviour (Benzer *et al.*, 1969; Hall et al., 1977). More recent techniques in genetics produce a different kind of mosaicism, known as expression mosaicism and have had a tremendous impact on behavioural studies.

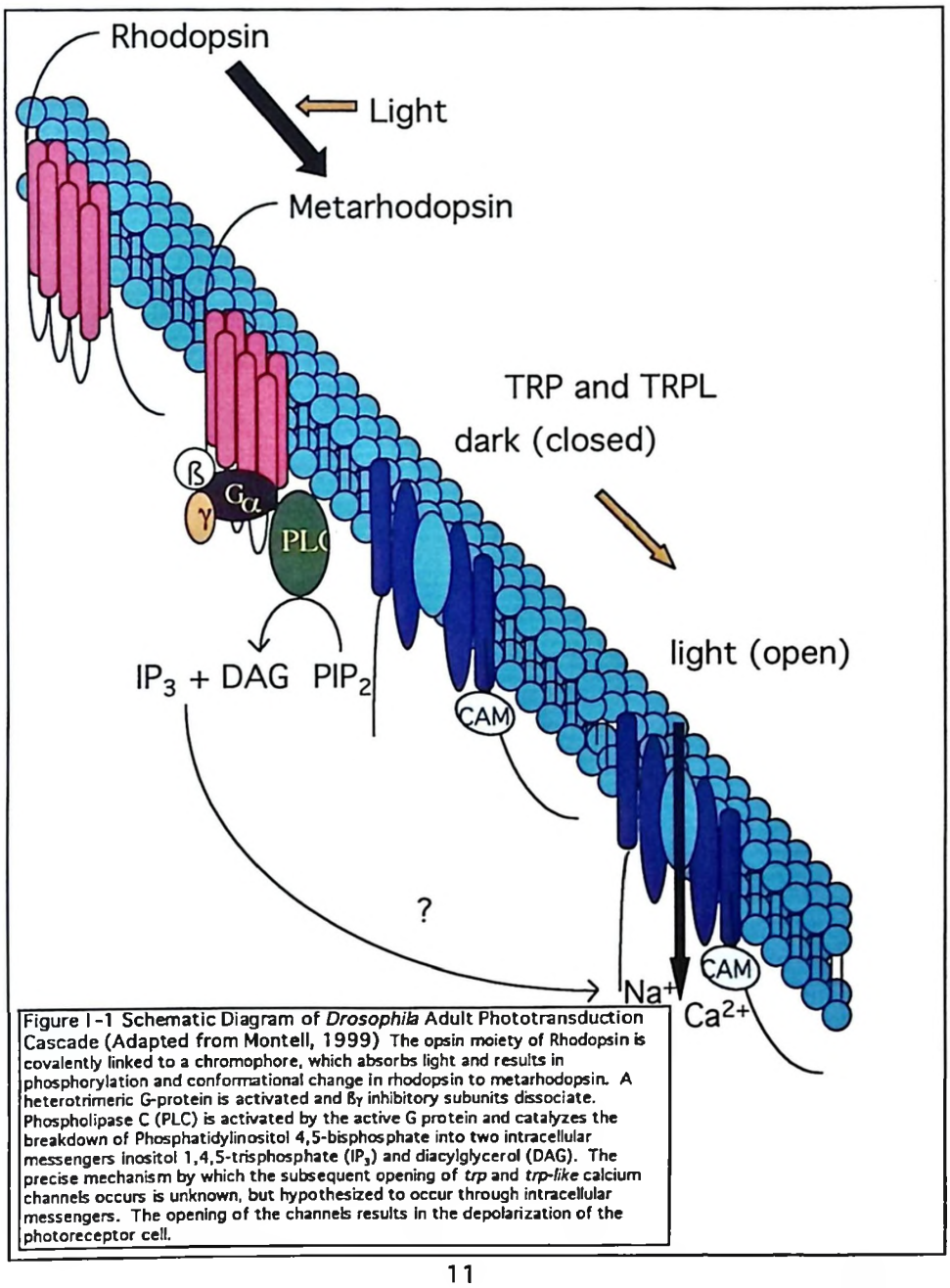

Figure I-1 Schematic Diagram of *Drosophila* Adult Phototransduction Cascade (Adapted from Montell, 1999) The opsin moiety of Rhodopsin is covalently linked to a chromophore, which absorbs light and results in phosphorylation and conformational change in rhodopsin to metarhodopsin. A heterotrimeric G-protein is activated and ßγ inhibitory subunits dissociate. Phospholipase C (PLC) is activated by the active G protein and catalyzes the breakdown of Phosphatidylinositol 4,5-bisphosphate into two intracellular messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The precise mechanism by which the subsequent opening of *trp* and *trp-like* calcium channels occurs is unknown, but hypothesized to occur through intracellular messengers. The opening of the channels results in the depolarization of the photoreceptor cell.

A variety of techniques have been employed to manipulate gene expression. One method requires target genes to be fused directly to a characterized promoter. Another common method is to activate genes under the control of a heat-shock promoter (hsp) (reviewed by Brand and Perrimon, 1994). The former method requires a change in promoter to alter spatiotemporal expression pattern. The latter circumvents this difficulty, offering the versatility in time and levels of expression through the application of temperature. However, the specificity of the expression is compromised, as expression from hsp tends to be ubiquitous (reviewed by Brand and Perrimon, 1994). The GAL4 system has emerged as a powerful technique, and has aided to overcome many of these problems (Brand and Perrimon, 1993). The system has been successfully used in misexpression and cell ablation analyses. It utilizes mobile genetic elements, known as P-element transposons, which makes it possible to introduce cloned genes into Drosophila and create transgenic lines.

The GAL4 technique has two main applications: enhancer detection technique and targeted gene expression directed by characterized promoters (Brand and Perrimon, 1993; reviewed in Brand and Dormand, 1995 and Phelps and Brand, 1998). In the case of enhancer detection, GAL4 can be expressed in a variety of different cell or tissue-specific patterns. The

insertion of a reporter gene into the P-element transposable elements has profound implications for behavioural studies permitting the correlation of expression patterns and behavioural effects. The second application of the system allows separate parental transgenic strains, one in which a characterized promoter is fused to *GAL4*, a transcriptional activator from yeast and another in which the UAS (Upstream Activation Sequence) is fused to the target gene, to be crossed (Brand and Perrimon, 1993). In the progeny, *GAL4* binds to the UAS promoter driving the ectopic expression of the target gene in specific cell-types, tissues or developmental stages (Figure 2.). The system has been of tremendous significance for targeted cell-ablation experiments (Hidalgo *et al.*, 1995; 1997; Sweeney *et al.*, 1995; McNabb *et al.*, 1997; Zhou *et al.*, 1997; Renn *et al.*, 1999) and also in labeling cells (Callahan *et al.*, 1994; Ito *et al.*, 1997).

The Checker Assay and ON/OFF Assay: Two Photobehaviour Assays which Facilitate a Genetic Analysis of the Larval Response to Light

Behavioural assays can be designed to study and genetically dissect larval photoresponse using single-gene mutations and cell ablations as tools to target individual photoreceptor cell types. The effects of light on *Drosophila* behaviour have been characterized and documented (Godoy-Herrera *et al.*,

1977; 1994). Third instar larvae exhibit characteristic behavioural patterns. Early in the developmental stage larvae display 'foraging' behaviour, as they burrow through the food substrate (Sokolowski *et al.*, 1984). At this particular time larvae show an aversion to light and display negative phototaxis (Sawin-McCormack *et al.*, 1995). However, towards the end of the third instar stage, when the larvae exhibits 'wandering' behaviour in search of pupation site, the aversion to light reverses (Godoy-Herrera *et al.*, 1994).

Two photobehaviour assays, the Checker Assay and the ON/OFF Assay, were designed with these characteristic larval responses to light, in mind (Busto *et al.*, 1999; Iyengar *et al.*, 1999; Hassan *et al.*, 2000). Both assays were designed to uncover the roles of various genes and cell-types in larval visual system function. Both the Checker Assay and the ON/OFF Assay are single-larva assays, which permit a large number of individuals to be tested in a relatively short period of time. In this respect, the assays have also facilitated large-scale genetic screening and the isolation and characterization of visual system mutants (Iyengar *et al.*,1999). The ON/OFF Assay permits the quantification of three discrete aspects of photobehaviour: modulation of locomotion in light versus dark, angle of direction change at light transitions and number of larval head swings in light versus dark. In the Checker assay, the larva is presented with a choice to remain in the dark

or light and a response is measured in terms of relative residence time in dark and light conditions.

As a starting point for a genetic dissection of Drosophila larval photoresponse, single-gene mutations in genes known to function in the adult phototransduction cascade have been utilized (Busto *et al.*, 1999). In this manner, correlations between the performance of various behaviours and gene function were ascertained. Genetic mutations, which perturb adult phototransduction, were found to disrupt subsets of light responses in the ON/OFF Assay (Busto *et al.*, 1999). In the first part of this thesis a similar analysis of genetic mutants was conducted in the Checker Assay. In the second part, photoreceptor cell-types were selectively ablated using the GAL4 system (Brand and Perrimon, 1993). The ON/OFF photobehaviour assay facilitated the analysis of Rh5 and Rh6 photoreceptor cells in mediating various aspects of larval response to light. An assessment of the potential secondary effects of ablation was also undertaken.

Chapter II: Materials and Methods

***Drosophila melanogaster* Culture:**

All *Drosophilla melanogaster* stocks were raised at 25°C in standard medium containing inactivated yeast, sucrose, agar, 10% tegosept in ethanol and acid mix (phosphoric acid and propionic acid) to prevent mold growth. Fresh active yeast was added to suppplement standard media. All test larvae were grown on plates (60 mm x 15 mm; Falcon, Becton Dickinson Labware) with standard media (as described above), supplemented with 1.25g of beta-carotene (Jamieson; 3mg/tablet) per liter and exposed to 12 hr light/dark cycles.

***Drosophila melanogaster* Stocks:**

In addition to wild type strains *CantonS* amd *OregonR*, the following *Drosophila* stocks were employed:

p [Rh5-gal4] (III) w⁻;Cyo/Sp;p[Rh5-*gal4*]/p[Rh5-*gal4*]

In this strain, a p-element containing the *gal4* gene, a transcriptional activator from yeast, is expressed under the Rh5 promoter. The opsin moiety of Rh5, is a short-wavelength absorbing in the blue region of the spectrum (Papatsenko *et al.*, 1997; Salcedo *et al.*, 1999). and structurally seven-transmembrane protein (Papatsenko *et al.*, 1997). Rh5 expression has been found in adult photoreceptor cells R8 (Papatsenko *et al.*, 1997), as well as the larval visual system (Desplan, personal communication).

pRh6-gal4 (III) w⁻;CyO/Sp;p[Rh6-*gal4*]/p[Rh6-*gal4*]

In this strain, a p-element carrying the Rh6 promoter fused to the yeast *gal4* gene. The *rh6* gene encodes the opsin moiety of Rh6 rhodopsin and is also

expressed in adult photoreceptor cells R8, in subsets of cells, which do not overlap with *rh5* expression (Papatsenko *et al.*, 1997). Rh6 is also expressed in the adult as well as larval system of *Drosophila* (Desplan, personal communication).

p[UASg-*hid*] (II) {X;+/+;p[UAS-*hid*]/p[UAS-*hid*]}

UAS (Upstream Activating Sequence), a promoter from yeast, contains a tandem array of five *GAL4* binding sites (Brand *et al*, 1985). The UAS promoter is fused to cell death gene *hid* (*head involution defective*) (Grether et *et al.*, 1995) and can drive cell-specific expression of *hid* in the presence of the transcriptional activator *GAL4* (Zhou *et al.*, 1997; McNabb *et al.*, 1997; Renn *et al.*, 1999).

p[UASg-*rpr*] (II) {X;+/+;p[UAS-*rpr*]/p[UAS-*rpr*]}

The *GAL4*-responsive promoter, UAS promoter is fused to cell death gene *reaper (rpr)* (White *et al.*, 1988) and drives the expression of the cell death gene in the presence of the transcriptional activator, *GAL4* (Zhou *et al.*, 1997; McNabb *et al.*, 1997; Renn *et al.*, 1999).

p[UAS-*tau-lacZ*] (III)

The *E.coli* reporter gene *lacZ* , which encodes the enzyme _-galactosidase is fused to the cDNA encoding bovine microtubule-binding protein, *tau* and Upstream Activation Sequence (UAS) promoter. The reporter gene can be expressed in the presence of *GAL4* in developing and adult neurons, thus labeling axons and cell bodies and assisting in their visualizaton (Callahan *et al.*, 1994).

Crosses

Female Rh5-*gal4* and Rh6-*gal4* strains were crossed to male UAS-*hid* and/or UAS-*rpr* strains. The F1 progeny were tested in the ON/OFF photobehaviour assay and the parental strains served as controls in the assay (see Appendix C. Cross Scheme).

Behaviour Assays: Collection of Larvae

Approximately, 200 adult flies aged 1-7 days were housed with fresh food plates (60 mm x 15 mm; Falcon, Becton Dickson Labware) and permitted to adjust to new environment for 48 hrs. A two hour egg pre-collection was conducted at 25°C using fresh food plates thinly coated with yeast paste. The pre-collection was followed up with a 1 hr collection, again on fresh food plates thinly coated with yeast paste. Eggs collected on media during 1 hr collection were incubated at 25°C and exposed to 12hr dark/light cycles. Between 20 and 22 hrs after egg lay (AEL), incubated plates were scrutinized under a dissection microscope for first instar larvae. These larvae were discarded and re-incubated at 25°C. After 1-2 hrs 25-50 newly hatched first instar larvae were identified and transferred to fresh food plates covered with a thin layer of yeast paste. Plates were incubated and third instar larvae were tested in photobehaviour assay between 84hr and 90hr (AEL)

ON/OFF Photobehaviour Assay

Test Surface

The pre-test and test plate consisted of a plastic petri dish (100 mm x 15 mm; Fisher Scientific Co.) containing 15 mL of 1% agarose cooled to room temperature. Since larvae prefer to stay in crevices, the surface had to be free of depressions. A circular test area with diameter of 90 mm was defined,

leaving a circular boundary of 10mm surrounding the test area. The boundary extended from the end of the test area to the edges of the plate, where the agar contacts the side of the plate. Any data collected beyond this boundary were discarded. Larvae were initially rinsed in distilled water for 5-10 seconds and then placed in the center of a designated pre-test plate with a moist paintbrush. Larvae were permitted to wander on the surface of the pre-test plate for 1 minute to adapt to the test conditions. The test plate itself rested on top of a light box and glass template with a 90 mm diameter circular region constructed with black adhesive tape. The template assisted in defining the boundaries of the test arena.

Light Sources

All manipulations were performed in the dark with red, dark room light (20W lamp with Kodak GBX-2 filter) providing illumination for videotaping purposes. *Drosophila* larvae do not respond to wavelengths greater than 650 nm, as verified in Hassan *et al.*, 1999 where Response Indices (R.I.) were around 0 in dark conditions. All manipulations were performed in the dark with dark room light (20W lamp with Kodak GBX-2 filter) providing illumination for recording purposes. A cool white fluorescent light bulb (20W Phillips) was used to irradiate the test plate from above. The lighting fixture had a spectral range spanning 400 to 650 nm wavelengths, with peaks at 440 and 560 nm. The intensity of the light was 1050 $\mu W/cm^2$ as measured with a Newport Digital Power Meter (Model 818)-SL. Irradiation time was carefully controlled with a timer programmed to turn the light fixture on and off resulting in alternating 10s dark and light pulses.

Videotaping and Automated Tracking

Larval movements were recorded and videotaped (Fuji HQ-120, RCA VCR) through the Fujinon TVZ zoom lens (Fuji Optical Co.) of a CCD TV camera (Elmo). The camera was also attached to a Power Macintosh 9500/200 Computer permitting the larval movements to be simultaneously visualized and tracked on an Apple Vision Monitor (Bala Iyengar, unpublished setup).

Data Analysis

Three different aspects of larval behaviour were quantified in the ON/OFF Assay: reduction of path length in light vs. dark pulses, change of direction of larval path at light transitions and increased frequency of head swinging in light vs. dark pulses.

A Response Index (R.I.) based on relative distances moved in the light versus the dark was calculated for each individual larva according to the following equation R.I. = dDARK - dLIGHT/dTOTAL. Throughout the course of the test the computerized System permitted simultaneous tracking of larval path during the course of the assay along with the computation of R.I's. A mean R.I.±SEM (Standard Error of Mean) was calculated for each strain. Larval paths were also traced (head tracings) from videotapes displayed on a video monitor (8" x 10" Hitachi 1-chrome). Tracings were then digitized using Apple OneScanner (100 dpi) and Mikrotek (100 dpi) and angles of path change at various light transitions were measured with NIH Image 161/PPC (downloaded from website http://rsb.info.nih.gov/nih-image) on PowerMac Computer System (PowerMacintosh G3). Again a mean direction change was calculated for each strain at Dark to Light (DL), Light to Dark (LD) and Dark to Dark (DD) transitions. Head swinging data were also obtained from

tracings and mean numbers of head swings per light and dark pulses were calculated.

Statistical Analyses

Minitab 10.5 for Macintosh and Microsoft Excel software were used in the statistical analysis of samples. On average sample sizes (n) were around 20. Statistical tests employed in the analysis of data included, Two-factor Analysis of Variances (ANOVA) and Tukey-Kramer multiple comparison tests (Minitab 10.5 for Macintosh). All data was normal and no transformation of data was necessary. All tests were performed at a level of significance of $\alpha=0.05$

Immunohistochemistry: m24B10 staining of Third Instar Larval Brains

Early foraging and late wandering third instar larval brains were stained using the m24B10 monoclonal antibody. Brains were dissected in 1X PBS buffer and then fixed overnight in 4% paraformaldehyde at 4°C. Brains were washed in PBS for 1hr with PBS changes every 10 minutes. Brains were subsequently immersed in blocking solution, which consisted of 150 uL of 0.2% Triton in PBS and 10 μL of NGS (Goat Serum) for 1hr. Old blocking solution was removed after 1hr and fresh blocking solution (quantities the same as previously described) along with 1 μL of the primary antibody m24B10 were added and brains were refrigerated at 4°C overnight. Larval brains were washed in 0.2% PBT for 2-3 hrs with solution changes every 10-20 minutes. Blocking solution was added as described above and then blocking solution with 1 μL of HRP (Horseradish Peroxidase)-conjugated secondary antibody was added and brains were kept at 4°C overnight. Larval brains

were then washed for 4-5 hrs with 0.2% PBT and solution changes were performed every 10-20 minutes. 500 µL of 1 mg/µL of DAB (Diaminobenzidene) was diluted with 500 µL of sterile H_2O. HRP enzymatic reaction was carried out. 200 uL or 0.5 mg/mL of DAB solution was added to larval brains and reaction was permitted to proceed for 1 minute. After 1 minute 6 µL of 3% H_2O_2 was added and solution swirled for 1-3 minutes. 1X PBS was added to stop the reaction and brains were washed in 1X PBS for 10 minutes. Larval brains were transferred and mounted in 70% Glycerol in PBS.

X-gal staining of Third Instar Larval Brains

Wandering third instar larval brains were dissected in 1X PBS and fixed in 4% paraformaldehyde (pH 7.4) for 15 minutes at room temperature. Brains were washed for 1hr in PBT (0.2% Triton-X) solution. 2% X-gal in DMSO was added to 1 mL of solution containing 10mM Na_2HPO_4·NaH_2PO_4 (pH 7.2), 150 mM $MgCl_2$, 3.1 mM $K_4Fe(CN)_6$, 0.3% Triton X-100. The solution was then heated on heating block at 65°C until clear. X-gal solution was permitted to cool at room temperature for a few minutes and then added to larval brains. Larval brains were incubated at 37°C overnight.

Preface

The work presented in this chapter was initiated a by previous Masters student, Macarena Busto and present Ph.D student, Balaji Iyengar, in the laboratory of Dr. Ana Regina Campos at McMaster University. M. Busto performed the locomotory analysis and the analysis of phototransduction mutants in the Checker Assay (Figures 4 and 6) and B. Iyengar the immunohistochemical and X-gal staining (Figure 7). My contribution to this chapter includes the measurement of response of two wild type strains at various light intensities as well as the analysis of mutations in genes which function in the development of the larval visual system (Figures 2, 3 and 5).

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Behavioral Characterization and Genetic Analysis of the *Drosophila melanogaster* Larval Response to Light as Revealed by a Novel Individual Assay

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A new assay was designed, named checker, that measures the individual response to light in the fruitfly *Drosophila melanogaster* larva. In this assay the *Drosophila* larva apparently modulates its pattern of locomotion when faced with a choice between a dark and lit environment by orienting its movement towards the dark environment. We show that, in this assay, a response to light can be measured as an increase in residence time in the dark versus the lit quadrant. Mutations that disrupt phototransduction in the adult *Drosophila* abolish the larval response to light, demonstrating that this larval visual function is similar to that of the adult fly. Similarly, no response to light was detected in strains where the larval visual system (photoreceptors and target area) was disrupted by a mutation in the homeobox containing gene *sine oculis (so)* gene. Ablation of photoreceptors by the targeted expression of the cell death gene *hid* under the control of the photoreceptor-specific transcription factor *glass (gl)* abolishes this response entirely. Finally, we demonstrate that this response to light can be mediated by rhodopsins other than the blue absorbing Rh1.

KEY WORDS: Insect; larval photobehavior; locomotion; *Drosophila;* behavioral mutants.

INTRODUCTION

The larval stages of the holometabolous insect *Drosophila melanogaster* has recently emerged as a model system for the genetic analysis of behavior (Monte *et al.*, 1989; Busto *et al.*, 1999; Kernan *et al.*, 1994; Osborne *et al.*, 1997; Park *et al.*, 1997; Tully *et al.*, 1994). Most of the larval development of *Drosophila melanogaster* is spent burrowed in the food substrate; a behavior described as foraging. During the last instar the larva ceases foraging and leaves the food substrate in search of an adequate site to undergo metamorphosis. Among the various behavioral changes that occur at this transition is the decrease in the larva's response to light in a population assay that assesses larval preference for a dark environment (Lilly and Carlson, 1990; Gordesky-Gold *et al.*, 1995; Sawin-McCormack *et al.*, 1995). Larval response to light has been detected in this population assay as early as the first instar (Sawin-McCormack *et al.*, 1995).

Little is known about the *Drosophila* larva visual system function and how larval locomotion is modulated by the light stimulus. Recently, using a novel individual assay we have demonstrated that a larval response to light can be measured as a reduction in the distance traveled, as a sharp change of direction and as an increase in head swinging (Busto *et al.*, 1999). Mutations that disrupt adult vision disrupt a subset of these responses suggesting the existence of a light perception function that does not utilize the same phototransduction cascade as the adult photoreceptors. Our results indicated that this novel visual function depends in part on the blue absorbing rhodopsin Rh1 and is specified by the *sine oculis (so)* gene (Busto *et al.*, 1999). However it may not reside in the larval photoreceptor organs, the so called Bolwig's

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organ (Bolwig, 1946; Steller *et al.*, 1987; Busto *et al.*, 1999).

From the morphological point of view the larval visual system of the *Drosophila* larva is very simple. It is comprised of two bilateral groups of twelve photoreceptor cells located anteriorly and juxtaposed to the mouth hooks similar to what is found in larger flies organ (Bolwig, 1946; Steller *et al.*, 1987). The axons of the photoreceptor cells form the larval optic nerve which innervates the optic lobe primordium area of the brain lobes. The early development and the establishment of connectivity in this system has been described in some detail (Green *et al.*, 1993; Campos *et al.*, 1995). The reported simplicity of the larval visual system is in contrast to the morphological complexity observed in the adult visual system. The main visual organ of the adult fly, the compound eyes, consist of circa 800 ommatidial units containing of 8 photoreceptor cells each. These photoreceptors project in a complex sterotypical pattern to the target area, the optic lobes in the brain (Meinertzhagen and Hanson, 1993).

In order to begin understanding the neural mechanisms underlying the regulation of larval locomotion by light we decided to take a genetic approach. Such an approach requires the isolation of single gene mutations to be used as tools in the identification of cell types and ultimately gene products required for the performance of the behavior under study. To that end appropriate assays need to be designed and the analysis of this behavior using previously isolated mutations need to be carried out in some detail. We reasoned that individual assays would be better suited for this purpose for the following reasons. First, it precludes the need of large numbers of synchronized larvae which is cumbersome in a mutant screen. Second, it allows the detection of other phenotypes (such as deficits in locomotion) that may interfere with the final response measured. Third, the progression of the assay can be recorded on video tape such that additional analysis of the larval behavior can be performed. Finally, the ability to test a behavioral phenotype in a single larva provides us with the tool to undertake a mosaic analysis where each individual is distinct regarding the distribution of mutant and wild type patches.

Two assays were designed that use single animals and where a per larva response index can be obtained. These assays, checker (this report) and the on/off (Busto *et al.*, 1999) are relatively quick. Each larva can be tested in less than 5 minutes making these assays suitable for large scale mutant screens.

Here, using the checker assay, we report that in the *Drosophila* third instar foraging larva the light stimulus modulates the direction of movement and that this response can be reliably measured in individual organisms. Mutations that disrupt phototransduction in the adult eye also disrupt this larval response. These results suggest that the larval and adult visual systems are similar from the functional point of view. The analysis of developmental mutants in which all or part of the larval visual system is missing, demonstrates that larval light detection measured by the checker assay is located in the larva's main visual organ the so called Bolwig's organ.

MATERIALS AND METHODS

Fly Stocks

Fly strains were grown at 25°C in 12 hr light/dark cycles on standard medium containing inactivated yeast, sucrose and agar supplemented with fresh active yeast and β carotene (1.25 g/l). Tegosept in ethanol and propionic acid were used to prevent mold growth. Strains used in addition to wild types *Canton-S* (*CS*) and *Oregon-R* (*OR*) are listed below:

Glass Multimer Reporter-Head Involution Defective (*pGMR-hid*). This strain contains a fusion vector in which the cell death gene *hid* is expressed under the control of the *gl* promoter (Grether *et al.*, 1995).

Neither Inactivation nor Afterpotential C (*ninaC*). The *ninaC* gene encodes two isoforms (3.6 and 4.8 kb RNA) of adult photoreceptor specific cytoskeleton proteins consisting of a protein kinase and a myosin head domain (Montell and Rubin, 1988). The $ninaC^5$ mutant allele used is a null that causes the reduction of the 3.6 and 4.8 kb RNA. $ninaC^5$ mutants show abnormal ERG, light and age-dependent retinal degeneration (Porter and Montell, 1993; Hofstee *et al.*, 1996) as well as a defect in response termination (Porter *et al.*, 1992).

Neither Inactivation nor Afterpotential E (*ninaE*). The *ninaE* germ encodes the opsin moiety of the Rh1 rhodopsin and is expressed in the adult photoreceptors R1–R6 (Serikaku and O'Tousa, 1994) as well as the larval visual system (Zuker *et al.*, 1985; Pollock and Benzer, 1988). The $ninaE^{17}$ mutation contains a 1.6 kb deletion. Mutant flies have very low rhodopsin levels and respond poorly to light stimulus (O'Tousa *et al.*, 1989).

No-Receptor Potential A (*norpA*). The *norpA* gene encodes a phospholipase C, which when absent, leads to a complete block of the phosphoinositide cascade

mediating phototransduction (Hardie and Minke, 1995). Adult flies lack light-elicited receptor potentials in the compound eyes and ocelli (Pak *et al.*, 1970). The *norpA*^{P24} contains a 28 base pair deletion in the coding region which produces a premature termination codon (Pearn *et al.*, 1996).

Sine Oculis (*so*). The *so* gene encodes a homeobox containing protein required for adult and larval visual system development (Fischbach and Technau, 1984; Serikaku and O'Tousa, 1994). The *so*^{mda} mutation prevents the development of the larval visual system (photoreceptors and target area) (Serikaku and O'Tousa, 1994).

Harvest of Synchronized Larvae

Adult flies aged from one to seven days were allowed to lay eggs in a fresh food plate (100 mm × 15 mm; Fisher Scientific) supplemented with vitamin A precursor (βcarotene, 1.25 g/L) and coated with yeast paste. After a minimum of two 2 hr precollections, a 1 hour egg collection was incubated at 25°C. At 20–22 hours after egg lay (AEL) all newly-hatched first instar larvae were removed under a dissection microscope. After a one-hour incubation period approximately 70 newly hatched first instar larvae were collected and transferred to a fresh food plate coated with yeast paste. Third instar larvae were tested for photobehavior at between 84–90 hours AEL.

Photobehavior Assay

Measurements of larval photobehavior were made on the checker assay. It consists of a plastic petri dish (100 mm × 15 mm; Fisher Scientific) containing 15 ml of 1% agarose cooled to room temperature. Irregularities on the agar surface affect larval behavior. *Drosophila* larvae like to remain in crevices. Therefore test plates need to be free of depressions (agar bubbles) and the test can not be performed near the edge of the plate where the agar touches the side of the plate. Thus a circular 1 cm boundary from the plate edge was established beyond which the data collected was discarded.

Manipulation of the larvae prior to the test was conducted using a dark room light (20 W lamp with Kodak GBX-2 filter). Testing was conducted using a cool white bulb with a spectrum of 400–650 nm with peaks at 440 and 560 nm (20W Cool White, Philips) and with a throughput of 1050 microwatts/cm^2. Light intensity was measured with a Newport Digital Power meter (Model 818-SL). The dark room light (20 W lamp with Kodak GBX-2 filter) used in this assay is the same employed to record circadian regulated locomotory behavior of *Drosophila* in free running conditions ("constant darkness") (Sehgal *et al.*, 1992). Larval photobehavior assays (Lilly and Carlson, 1990; Busto M.Sc. thesis, 1999) conducted using the dark room light as the sole light source yielded response indices close to zero confirming previous reports that *Drosophila* does not respond to light stimulus above the 650 nm range (Ashburner, 1989).

Individual larvae were removed, using a moist paintbrush, from the culture dish. Each larva was carefully rinsed with distilled water to remove any excess food particles, removed from the distilled water using a flathead paintbrush, and placed on a pre-test plate for a period of 1 minute to allow for acclimatization to the agar surface. Each larva was then positioned in the center of the test plate. Each plate was positioned on a template consisting of 1 cm squares constructed in a checker board manner using black adhesive tape on the upper surface of the glass plate. The dark squares block out light while the light squares permit light transmission. Template and dish were positioned on a light box that was modified to emit light only in an 11 cm diameter. Template and dish were lit from below by a light box.

Temperature

Surface temperature recordings were taken in 25 sec intervals for 200 sec during the course of the checker assay using a 21X Micrologger (Campbell Scientific Ltd.). Temperature readings in either the checker assay (light or dark checks), or under safe light conditions were 21.5 ± 0.5°C (data not shown).

Data Collection and Analysis

Larval movement was visualized through a Fujinon TV - Z zoom lens (Fuji Optical Co.) attached to a CCD TV camera (Elmo TSE 272S) and recorded on videotape (Fuji HQ-120, RCA VCR). Larval behavior was recorded either until they reached the 1 cm boundary or total test time (180 seconds) had elapsed. Data derived for each of the strains was obtained from two to three sets of samples in which approximately ten larvae were tested each time.

Residence time in the dark and light quadrants were obtained using the VCR timer and started 5 seconds after the larva was placed in the center of dark

checker. Response indices (R.I.) [(time in dark − time in light)/total time of test or path length per cycle], with lights on ($R.I.^{on}$) and without lights ($R.I.^{off}$) were calculated on a per larva basis and an average of these individual indices was taken. A response to light is present when the R.I. with the light on is significantly higher ($p < 0.05$) than that obtained in the absence of light. In most experiments the same larva was tested with and without light (see below). Two standard wild type strains (CS and OR) showed a response to light whether the same or a different set of larvae were assayed with or without light. The data are depicted as means plus or minus standard error ($X \pm SEM$). The data, shown in Figs. 2, 3, and 5, were analyzed with paired T tests (two-tailed) at a level of significance of $\alpha = 0.05$. Part of the data shown in Fig. 4 was analyzed with paired T tests (*ninaE*^{17}), the rest of the data shown in this figure and Fig. 6 were analyzed with two-sampled T-tests at the same level of significance.

Immunohistochemistry

Larval brains with the eye-antenna imaginal discs were dissected from third instar larvae in PBS (Phosphate Buffer Saline), and fixed in 4% paraformaldehyde (pH 7.4) for 45 min at room temperature. This was followed by 3–4 PBS washes. Brains were incubated in a blocking solution, which contained PBS with 0.3% Triton X-100 and 5% goat serum at room temperature for 1 hr, followed by addition of a fresh 100 µl of blocking solution containing 2 µl of 24B10 primary. After 8–12 hrs of incubation at 4°C the samples were washed with PBT (phosphate Buffer Triton) for 4 hrs, with changes every 10–20 min, followed by incubation in blocking solution as described above and finally incubated with a secondary antibody conjugated to horseradish peroxidase for another 8–12 hrs. Specimens were washed thoroughly for 4 hrs and stained with 0.5 ml of 3, 3′-diaminobenzidine (0.5 mg/ml, Sigma 72H3614) in the presence of hydrogen peroxide. The reaction was stopped by washing several times with PBS. Samples were mounted in 70% glycerol in PBS, and observed with a Zeiss axiophot microscope.

RESULTS

Larval Response to Light Can be Measured as Increased Residence Time in Dark

The checker assay follows the general design of the original plate assay (Lilly and Carlson, 1990) except that smaller quadrants and individual larvae are tested. Observations of larval behavior during the course of this assay suggest that the wild type larva remain preferentially in the dark environment. As the larva encounters a light quadrant it retracts its head and returns to the original dark environment. It is seen often with more than half of its body length positioned over the dark/light boundary. Even in this situation the larva is often able to return to the original position of the dark checker (Fig. 1A, frame 00:03).

While the larva is in the lit quadrant the path shape is typically straight (Fig. 1B). While in the dark quadrant the path shape is often convoluted, likely reflecting an attempt to remain in the dark quadrant and/or a repulsion to light (Fig. 1B). Therefore, in this assay, path shape or the modulation of locomotion in the two environments is apparently a reflection of the larva's attempt to remain in the dark environment (i.e. light avoidance when confronted with a dark/light boundary).

In an attempt to quantify these observations two response indices (R.I.), with the light on ($R.I.^{on}$) and with the light off ($R.I.^{off}$), were calculated for each larva. The response index is based on residence time in dark and light quadrants (R.I. = (total time in dark quadrants − total time in light quadrants)/total (test time)). Thus a response to light in this test is represented by a significant effect of light on the R.I. The R.I.'s obtained with the light on and off were significantly different in the two wild type strains tested indicating an effect of light on larval behavior (Fig. 2).

In a mutant screen, lack of response may be due to reduction in sensitivity that may represent a specific phenotype. Alternatively, variability in the sensitivity of larvae due to variables in food and rearing conditions may reduce its ability respond to a light stimulus in this assay. In order to determine how sensitive the larval visual system is in this assay we used neutral density filters to reduce the light throughput in the lit quadrants in a stepwise fashion thereby reducing the difference between the lit and dark quadrants. The two wild type strains used (OR and CS) were able to respond in this assay even when the light throughput of the lit quadrant was only 14.3% of the original test condition (Fig. 2 and 3). However, upon further reduction of light, the CS strain was unable to respond in this assay while the OR strain responded normally. These results demonstrate that the conditions of this assay allow us the detection of a larval response to light above the threshold of variations in light sensitivity of two standard wild type strains.

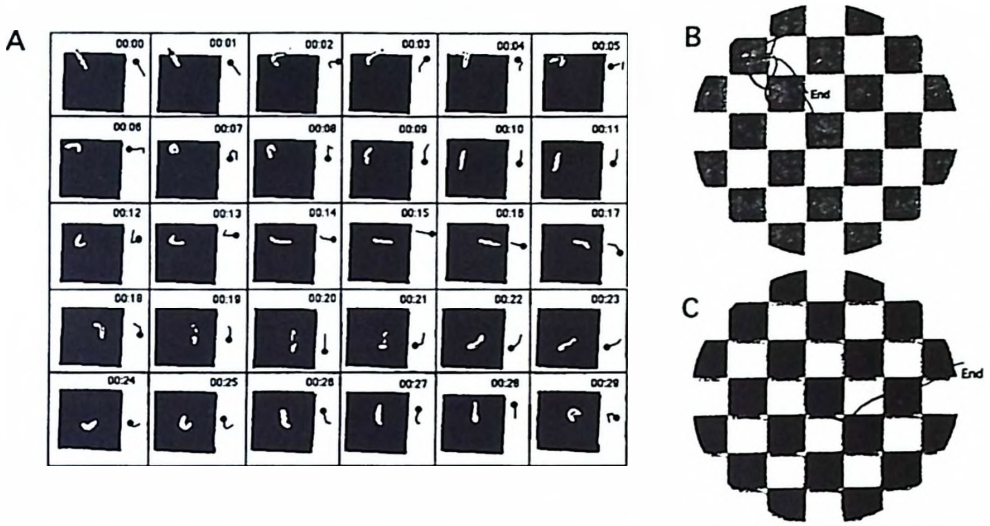

Fig. 1. Larval behavior in the checker assay. A-Video of a single *CS* larva tested in the checker assay was used to generate frame by frame photographs. On the right of each panel a schematic drawing depicts the relative position of the head (filled circle) and body (line). Frames 0 to 7 show a larva that, as it approaches the dark/light boundary, reacts by retracting its head (frames 2 to 4) and then returns to the dark square by making a 180° turn towards the opposite direction (frames 5 to 9). Frames 10 to 29 show the larva circling within the dark check without approaching the light/dark boundary. B-Diagramatic representations of path taken by a wild type *CS* larva in both test (Light) and control (Dark) conditions.

The Light Response Measured in the Checker Assay Is Mediated by a Phototransduction Pathway Similar to Adult Visual System Function

In order to determine whether this assay was detecting larval visual function similar to the one previously described for adult flies, larvae carrying null mutations in *norpA*, *ninaC* and *ninaE* genes were tested as described above. These genes are required in the adult fly for visual system function and for phototransduction in the compound eye (reviewed by Ranganathan *et al.*, 1995).

The *ninaC* gene encodes two retina-specific chimeric proteins consisting of a protein kinase and a myosin head domains (Montell and Rubin, 1988). One of these a 132 kD protein (p132), is expressed primarily in the cytoplasm. The other, a 174 kD protein (p174) is localized predominantly in the rhabdomere (Porter *et al.*, 1992; Porter and Montell, 1993). The $ninaC^5$ mutant allele has reduced levels of both p132 and p174.

The *norpA* gene encodes a phospholipase C, an essential component of the phototransduction signaling cascade in the adult eye (Bloomquist *et al.*, 1988; Ranganathan *et al.*, 1995). The *norpA* gene is expressed as two developmentally-regulated transcripts (subtypes I and II) generated by alternative splicing (Kim *et al.*, 1995). Subtype I is specific to the adult eye while subtype II is found in the CNS of adults and larvae (Kim *et al.*, 1995). Therefore, disruption in the response to light in larvae carrying a null allele of the *norpA* gene may be due to lack of this gene's function in the larval CNS and/or larval visual system.

The values obtained for $R.I.^{on}$ and $R.I.^{off}$ were not significantly different in larvae homozygous mutant for the *norpA* and *ninaC* genes indicating that mutations in these genes abolish the larval response to light as measured in this assay (Fig. 4). In contrast, lack of the blue absorbing rhodopsin Rh1 had no effect on the larval response to light suggesting that in this assay, larval response can be mediated by photoreceptors expressing other rhodopsins (Fig. 4).

Mutations that severely disrupt locomotion cannot be tested in the checker assay (Iyengar *et al* 1999).

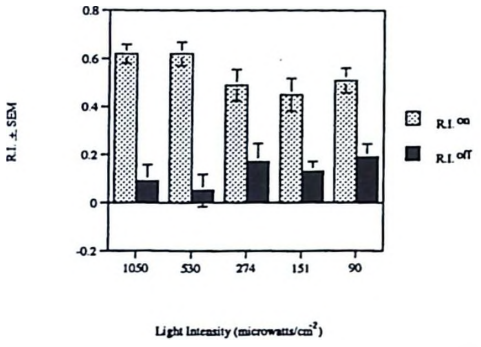

Fig. 2. Response of the wild type strain OR in the checker assay at various light intensities. OR foraging third instar larvae were tested in five different light intensities spanning the range from 1050 microwatts/cm² to 90 microwatts/cm². Two R.I. were calculated per larva: in the presence ($R.I.^{on}$) and in the absence ($R.I.^{off}$) of light. A significant difference between $R.I^{on}$ and $R.I.^{off}$ represents a response to light. Paired t-tests were used to determine significancy ($p < 0.05$). Significant differences were calculated for all light intensities: 1050 microwatts/ cm² ($n = 20$, $p < 0.001$), 530 microwatts/ cm² ($n = 20$, $p = 0.001$), 274 microwatts/ cm² ($n = 20$, $p < 0.0024$), 151 microwatts/ cm² ($n = 20$, $p = 0.001$), 90 microwatts/ cm² ($n = 20$, $p < 0.001$).

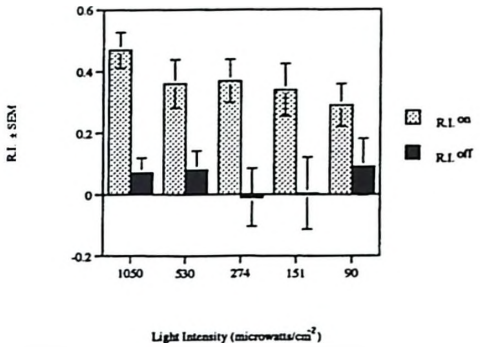

Fig. 3. Response of the wild type strain CS in the checker assay at various light intensities. CS foraging third instar larvae were tested in five different light intensities spanning the range from 1050 microwatts/ cm² to 90 microwatts/ cm². Two R.I. were calculated per larva: in the presence ($R.I.^{on}$) and in the absence ($R.I.^{off}$) of light. A significant difference between $R.I^{on}$ and $R.I.^{off}$ represents a response to light. Paired t-tests were used to determine significancy ($p < 0.05$). Significant differences were found for four light intensities: 1050 microwatts/ cm² ($n = 25$, $p < 0.001$), 530 microwatts/ cm² ($n = 20$, $p = 0.0069$), 274 microwatts/ cm² ($n = 20$, $p < 0.001$), 151 microwatts/ cm² ($n = 15$, $p = 0.014$). No significant difference between $R.I.^{on}$ and $R.I.^{off}$ was found when CS larvae were tested at 90 microwatts/ cm² ($n = 20$, $p = 0.14$).

In order to determine whether locomotion was affected by any of the mutations used in this paper we measured the distance traveled in 30 sec in the absence of light. These were compared to the two wild type strains used as baseline for the response to light measured in the checker assay (CS and OR). Locomotion in these wild type strains is significantly different, with OR displaying less locomotion than CS (Fig. 6). Their response to light is however indistinguishable, i.e., $R.I.^{on}$ is significantly different from $R.I.^{off}$ in both strains. These observations suggest that locomotion which is within the range exhibited by CS and OR strains does not affect the response to light evaluated in the checker assay. All the mutant strains used here except for one (*pGMR-hid*) display locomotion within the range established for OR and CS wild type strains. (Fig. 6). The locomotion measured for *pGMR-hid* mutant larvae is significantly lower that that shown for OR and this strain does not respond to light (below and Fig. 6). This result could be interpreted as an inability of *pGMR-hid* mutant larvae to respond to light due to reduced locomotion. However, several other strains with locomotion measurements indistinguishable from that calculated for *pGMR-hid* mutant larvae have been shown to respond to light in this assay (Iyengar *et al* 1999). We concluded that the lack of response seen in *pGMR-hid* mutant larvae is due to a defect in the visual system and not a significant effect on locomotion.

Ablation of the Bolwig's Organ and the Optic Lobe Primordium Disrupts the Larval Response to Light

In *D. melanogaster*, the larval visual system is comprised of two bilateral groups of twelve photoreceptor cells located anteriorly and juxtaposed to the mouth hooks similar to what is found in larger flies (Steller *et al.*, 1987). These photoreceptors project posteriorly and ventrally around the brain hemispheres terminating in the optic lobe primordium (Campos *et al.*, 1995; Green *et al.*, 1993; Schmucker *et al.*, 1997). The *so* gene encodes a homeodomain protein expressed in the optic lobe primordium and in the developing larval photoreceptors during embryogenesis and in the developing adult visual system (photoreceptor cells and optic lobes) in larvae (Cheyette *et al.*, 1994; Serikaku and O'Tousa, 1994). *so* functions include,

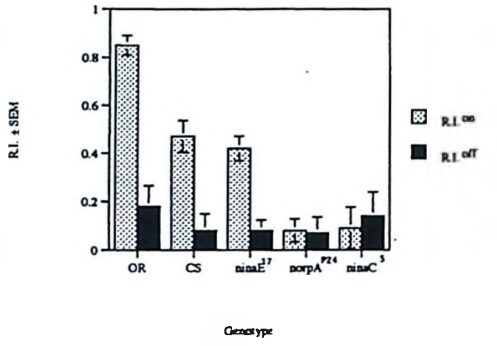

Fig. 4. Response in the checker assay of wild type larvae and larvae with mutations in genes required for adult phototransduction. Two R.I. were calculated: in the presence ($R.I.^{on}$) and in the absence ($R.I.^{off}$) of light. A significant different between $R.I^{on}$ and $R.I.^{off}$ represents a response to light ($p < 0.05$). The genotypes tested in this assay are: wild type strains CS (light on, n = 30; light off, n = 20) and OR (light on, n = 18; light off, n = 20) as well as the phototransduction mutants $norpA^{P24}$ (light on, n = 29; light off, n = 30), $ninaC^5$, (n = 20) and $ninaE^{17}$, (n = 19). $R.I^{on}$ and $R.I.^{off}$ for wild type strains CS and OR and $ninaE^{17}$, mutant larvae are significantly different (CS, $p < 0.001$; OR, $p < 0.001$, $ninaE^{17}$ $p < 0.001$). In contrast, the $R.I^{on}$ and $R.I.^{off}$ for the phototransduction mutants $norpA^{P24}$ and $ninaC^5$ are not significantly different ($norpA^{P24}$, $p = 0.87$, $ninaC^5$, $p = 0.69$).

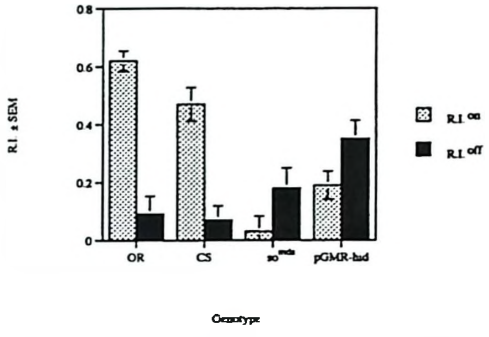

Fig. 5. Response in the checker assay of wild type strains CS and OR and the visual system mutants, *pGMR-hid* and so^{mda}. Two R.I. were calculated: in the presence ($R.I.^{on}$) and in the absence ($R.I.^{off}$) of light. A significant difference between $R.I^{on}$ and $R.I.^{off}$ represents a response to light ($p < 0.05$). The $R.I^{on}$ and $R.I.^{off}$ for both wild type strains OR (n = 20, $p < 0.001$) and CS (n = 25, $p < 0.001$) are significantly different. The $R.I^{on}$ and $R.I.^{off}$ for the visual system mutants so^{mda} (n = 20, $p = 0.13$) and *pGMR-hid* (n = 30, $p = 0.064$) are not significantly different.

regulating genes necessary for proper optic lobe invagination and Bolwig's organ formation during embryogenesis (Serikaku and O'Tousa, 1994). In order to determine whether the larval visual system, as defined by the so gene function, mediate the response to light measured here, larvae carrying a mutation in the so gene were assayed.

The so^{mda} mutant allele prevents the formation of the optic lobe placode that gives rise to photoreceptors and optic lobe primordium of the larval and adult visual system (Serikaku and O'Tousa, 1994). Therefore these strains lack not only the photoreceptor cells but also the target area which in the embryo is called the optic lobe primordium. No significant response to light was detected in so^{mda} mutant larvae demonstrating that the visual function that mediates the response to light measured in the checker assay resides in cells that require the so gene function (Fig. 5).

Next we sought to determine whether the photoreceptor cells, defined as the retinular cells that require the zinc finger transcription factor *gl* for differentiation, are the main photosensory organ required for this light response. To that end a strain in which a cell death gene (*hid*) is under the control of the *gl* promoter (*pGMR-hid*), was analyzed (Grether *et al.*, 1995). In adult flies ectopic expression of the *hid* gene in the developing adult photoreceptors is sufficient to ablate these cells (Grether *et al.*, 1995). Larvae carrying two copies of the *pGMR-hid* construct did not respond to light when tested in the checker assay (Fig. 5).

gl is expressed in the larval and adult photoreceptor neurons and in two groups of approximately 21 neurons in each brain hemisphere (Moses *et al.*, 1989). Not all cell types are equally sensitive to ectopic expression of cell death genes (H. Steller, personal communication). Therefore lack of response of the *pGMR-hid* larvae in the checker assay could be due to ablation of all *gl*-expressing cells or of a subset of these cells. For example, the larval retinular cells may not have been entirely killed by the ectopic expression of the cell death gene *hid*, suggesting that the complete set of retinular cells are required for the light response being measured in the checker assay.

In order to address these questions the integrity of *gl*- expressing cells in *pGMR-hid* larvae was estimated by labeling larval brains with the monoclonal antibody 24B10 that detects a photoreceptor—specific protein (Zipursky *et al.*, 1984). A monoclonal antibody that recognizes the *gl* gene product (Moses *et al.*, 1989) and

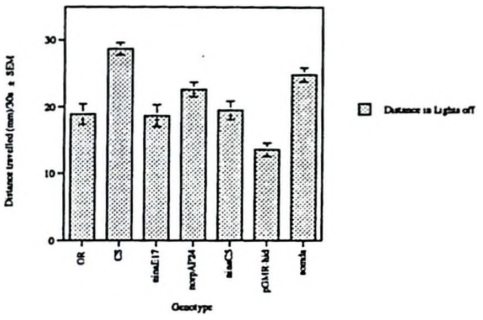

Fig. 6. Locomotion of OR, CS and of the mutant strains used. The graph depicts the mean distance traveled (SEM) in 30 seconds on a non-nutritive substrate in the absence of light. The locomotion measured for the OR wild type strain (n = 22) is significantly lower than that of *norpA*^{P24} (n = 30; p = 0.048) and of *sunb* (n = 20, p = 0.0033) and significantly higher that that of *pGMR-hid* (n = 31, p = 0.0038). It is indistinguishable from *ninaC*^{5} (n = 16, p = 0.78) and *ninaE*^{17} (n = 20, p = 0.91). The locomotion of the CS wild type strain is significantly higher than all mutant strains tested (*norpA*^{P24} p < 0.001; *sunb* p = 0.0033; *pGMR-hid* p < 0.001; *ninaC*^{5} p < 0.001; *ninaE*^{17} p < 0.001).

thus also other *gl*-expressing cells besides the retinular cells was also used in *pGMR-hid* strains. Additionally, larval brains from p*GMR-hid* larvae also carrying the *pGMR-lacZ* reporter construct were dissected and labeled with X-Gal histochemistry. The advantage of using the expression the *lacZ* gene under the control of the *gl* promoter is that the reporter (*lacZ*) can be found in the axons as well as cell bodies while the *gl* gene product is found only in the nuclei.

No labeling with the 24B10 monoclonal antibody was detected in *pGMR-hid* brains indicating that all larval photoreceptors were either absent or severely damaged by the ectopic expression of the cell death gene *hid* (Fig. 7B). Similar conclusions were reached when the expression of the *pGMR-lacZ* reporter construct was used to identify the larval photoreceptor cells (data not shown). Surprisingly, the *gl*-expressing central brain neurons were apparently not affected by the expression of the cell death gene *hid* as seen by the normal appearance of the *lacZ* expression (cell bodies and projection pattern) and of the expression of *gl* gene product (Fig. 7C to E). We concluded that the cell death gene *hid* is unable to affect other *gl*-expressing cells besides the larval retinular cells demonstrating that the absence of a light response in the *pGMR-hid* strain is due to absence of Bolwig's organ.

DISCUSSION

The *Drosophila* larval response to light represents a simple and easily quantifiable behavior likely to include components of more complex behaviors executed by higher organisms. *Drosophila* as, a model system, provides high resolution genetic and molecular biology tools to dissect the components, molecular and cellular, required for larval response to light (Miklos and Rubin, 1996).

In order to initiate a genetic dissection of larval response to light an assay was designed that tests a single larva. This assay is fundamentally different from the previously reported on/off assay as it makes different demands upon the larva (Busto *et al.*, 1999), see below. Therefore, it can be used to identify disruptions in different aspects of larval behavior.

In the checker assay the larva responds to light by performing complex maneuvers in order to remain in the preferred dark environment. Consequently, the pattern of larval locomotion in the dark is consequence of its avoidance of the light quadrant. A response to light in this assay is measured as an increase in residence time in the dark quadrant over that of the lit quadrant. Our results demonstrate that the larval response to light being measured in the checker assay is mediated by the Bolwig's organ and its first order neurons located in the optic lobe primordium. Ablation of Bolwig's organ only by the targeted expression of the cell death gene *hid* is sufficient to completely abolish the response to light measured in this assay. This is in contrast with previous findings using the on/off assay (Busto *et al.*, 1999). During the course of this assay the individual larva is subjected to intermittent pulses of light and dark (10 sec) and its behavior is recorded. The path tracing derived from individuals is used to measure various locomotory parameters such as change of direction at the transition between light and dark as well as path length, and head swinging frequency during the light and dark pulses. Analysis of these behaviors in wild type strains demonstrated that light modulates locomotion as seen by the reduction in the distance traveled that occurs during the light pulse. Additionally, turning on the light triggers a change in the direction of the larval path greater than when the light is turned off which in turn is greater than that measured in the absence of any light transition. Ablation of Bolwig's organ abolishes only the increased change of direction triggered by the dark to light transition (Busto *et al.*, 1999). These observations support the notion that an extra ocular light detection function exists that per-

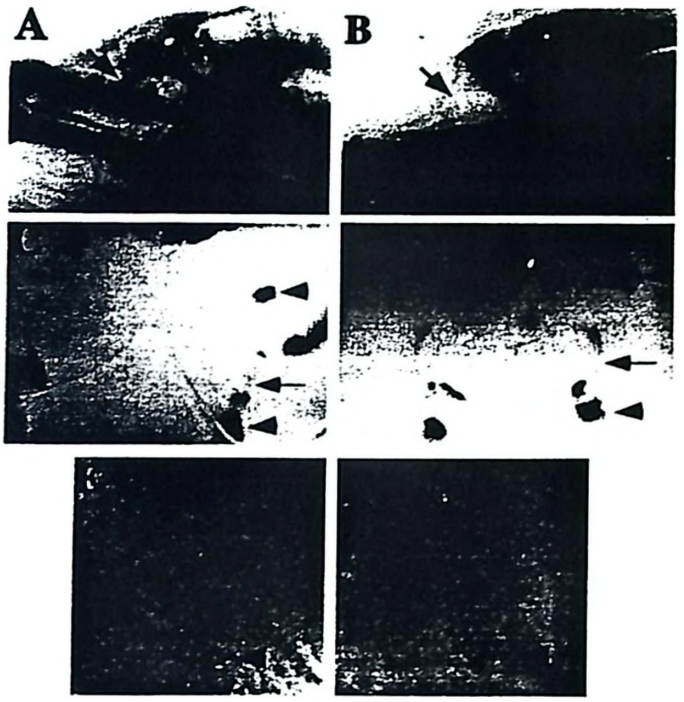

Fig. 7. Visualization of *gl*- expressing cells in *pGMR-hid* larvae. Whole mount immuno histochemistry of mouth hooks (A, B) where the Bolwig's organ is located and central nervous systems (C-F) dissected from third instar larvae. A minimum of 8 brain hemispheres were analyzed from each mutant strain. The monoclonal antibody 24B10 which labels the membrane of all retinular cells was used to visualize Bolwig's organ: (A) Wild type specimen showing the cell bodies (arrow) and the larval optic nerve (arrowhead), (B) *pGMR-hid* specimens do not show any 24B10 labeling (arrow). The expression of the *pGMR-lacZ* reporter construct as seen by βgalactosidase activity was used to identify gl-expressing cells in the central nervous system of third instar larvae. The pattern of βgalactosidase activity, which labels both the cell bodies (arrowheads in one brain hemisphere in C and D) and the axonal projections (arrows in one brain hemisphere in C and D), is the same in wild type (C) and *pGMR-hid* larvae (D). The anti-Glass antibody was also used to visualize the central nervous system *gl*-expressing neurons in wild type (arrowheads in E) and *pGMR-hid* larvae (arrowheads in F). No difference was observed between specimens of these two strains.

ceives the presence of light transitions but which cannot distinguish between the light being turned on from off (Busto *et al.*, 1999).

The light response measured by the checker assay can be mediated by rhodopsins other than the blue absorbing Rh1 encoded by the *ninaE* gene. This observation is similar to that found in the on/off assay (Busto *et al.*, 1999). These results do not exclude the potential role of this rhodopsin in mediating the light response detected in the checker assay. This question can only be addressed by the spectral sensitivity analysis of this behavior.

No statistically significant difference was found between the R.I.^{on} and the R.I.^{off} for the so^{mda} and *pGMR-hid* mutant strains indicating that these mutant strains do not respond to the light stimulus in this assay. However, in these two strains the R.I.^{off} was slightly higher than the R.I.^{on}. A possible explanation for this observation is that a residual light perception exists that cannot discriminate between the dark (preferred) and the lit (repulsive) environments but which nevertheless propels the larva forward. In the absence of light (test run in complete darkness) the larva remains in the dark quadrant, the location where it was placed at the

beginning of the test, which leads to a slightly higher, but non significant, R.I..

The locomotory reaction of organisms to biotic or abiotic factors has been traditionally defined relative to the source of stimulus (Fraenkel and Gunn, 1961). In a directed reaction (taxis), movement is modulated in order to position the long axis of the organism towards or away from the source of stimulation. In undirected locomotory reactions (kinesis), quantitative aspects of locomotion such as speed and frequency of turning are modulated by the stimulus.

These definitions can be further refined when the stimulus is varied temporally and quantitatively. In klinotaxis orientation is achieved by comparison of the stimulus intensity over time while in tropotaxis the differential stimulation of paired receptors in space orients the animal relative to the stimulus source. Thus in taxis the movement of the organism during orientation reflects the different way in which the stimulus is perceived. So for example, in klinotaxis orientation is indirect resulting from the alternating movements of the body necessary for the temporal measurement of the stimulus intensity. The resulting path is wavy with its overall direction towards or away from the stimulus source. In tropotaxis no deviations of the path are observed because the relative intensity of the stimulus is perceived in space due to the unequal stimulation of bilateral receptors. Thus, in this case the resulting path is straight towards or away from the source (Fraenkel and Gunn, 1961).

Using the checker assay, we show that the *Drosophila* larva is able to orient its body axis away from the light source, a behavior defined as taxis. This behavior, during the course of the assay, results in an increase in the residence time in the dark quadrant over that recorded for the lit quadrant. Whether the comparison of relative light intensity is being performed by the larval visual system temporally (klinotaxis) or spatially (tropotaxis) was not directly addressed by the experiments described in this paper. The observation that the larva upon entering a lit quadrant will, most of the time, return to its previous position in the dark suggests that a measurement of light was performed over time, a characteristic of the behavior described as klinotaxis (Fraenkel and Gunn, 1961). However, the presence of paired photoreceptors (the Bolwig's organ) and the fact that the larval path through the lit quadrant is straight, strongly suggest that the behavior being observed in this assay fits instead the classical definition of tropotaxis (Fraenkel and Gunn, 1961). Therefore, the checker assay described here measures an aspect of the *Drosophila* larval response to light fundamentally different from the one measured by the on/off assay (Busto *et al.*, 1999). The latter measures the modulation by light of quantitative aspects of locomotion such as speed and frequency of turning; a behavior defined as kinesis (Fraenkel and Gunn, 1961).

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REFERENCES

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

Bloomquist, B. T., Shortridge, R. D., Schneuwly, S., Perdew, M., Montell, C., Steller, H., Rubin., G., and Pak., W. L. (1988). Isolation of a putative phospholipase C gene of *Drosophila, norpA*, and its role in phototransduction. *Cell* 54:723–733.

Bolwig, N. (1946). Sense and sense organs of the anterior end of the house fly larvae. *Vidensk. Medd. Dan. Naturhist. Foren.* 109: 81–217.

Busto, M., Iyengar, B., and Campos, A. R. (1999). Genetic dissection of behavior: Modulation of locomotion by light in the *Drosophila melanogaster* larva requires genetically distinct visual system functions. *J. Neurosci.* 19:3337–3344.

Campos, A. R., Lee, K. J., and Steller, H. (1995). Establishment of neuronal connectivity during development of the *Drosophila* visual system. *J. Neurobiol.* 28:313–329.

Cheyette, B. N. R., Green, P. J., Martin, K., Garren, H., Hartenstein, V., and Zipursky, S. L. (1994). The *Drosophila sine oculis* locus encodes a homeodomain-containing protein required for the development of the entire visual system. *Neuron* 12: 997–996.

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

Fraenkel, G. S., and Gunn, D. L. (1961). *The Orientation of Animals*, Dover, New York.

Gordesky-Gold, B., Warrick, J. M., Bixler, A., Beasley, J. E., and Tompkins, L. (1995). Hypomorphic mutations in the larval photokinesis A (lphA) gene have stage-specific effects on visual system function in *Drosophila melanogaster*. *Genetics* 139: 1623–1629.

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

Grether, M. E., Abrams, J. M., Agapite, J., White, K., and Steller, H. (1995). The *head involution defective* gene of *Drosophila melanogaster* functions. *Genes Dev.* 9:1694–1708.

Hardie, R. C., and Minke, B. (1995). Phosphoinositide-mediated phototransduction in Dorosophila photoreceptorsd: The role of Ca2+ and trp. *Cell Calcium* 18:256–274.

Hofstee, C. A., Henderson, S., Hardie, R. C., and Stavenga, D. G. (1996). Differential effects of *ninaC* proteins (p132 and p174)

on light-activated currents and pupil mechanisms in *Drosophila* photoreceptors. *Visual Neurosci.* 13:897–906.
Kernan, M., Cowan, D., and Zuker, C. (1994). Genetic dissection of mechanosensory transduction mechanoreception- defective mutations of *Drosophila*. *Neuron* 12:1195–1206.
Kim, S., McKay, R. R., Miller, K., and Shortridge, R. D. (1995). Multiple subtypes of phospholipase C are encoded by the *norpA* gene of. *J. Biol. Chem.* 270:14376–14382.
Lilly, M., and Carlson, J. R. (1990). *Smellblind:* A gene required for *Drosophila* olfaction. *Genetics* 124:293–302.
Meinertzhagen, I. A., and Hanson, T. E. (1993). The development of the optic lobe. In Bate, M., and Arias, A. M., (eds.), The Development of *Drosophila melanogaster*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 1363–1482.
Miklos, G. L. G., and Rubin, G. M. (1996). The role of the genome project in determining gene function: Insights from model organisms. *Cell* 86:521–529.
Monte, P., Woodard, C., Ayer, R., Lilly, M., Sun, H., and Carlson, J. R. (1989). Characterization of the larval olfactory response in *Drosophila* and its genetic basis. *Behav. Genet.* 19: 267– 283.
Montell, C., and Rubin, G. M. (1988). The *Drosophila ninaC* locus encodes two photoreceptor cell specific proteins with domains homologous to protein kinases and the myosin heavy chain head. *Cell* 52:757–772.
Moses, K., Ellis, M. C., and Rubin, G. M. (1989). The *glass* gene encodes a zinc-finger protein required by *Drosophila* photoreceptor cells. *Nature* 340:531–536.
Osborne, K. A., Robichon, A., Burgess, E., Butland, S., Shaw, R. A., Coulthard, A., Pereira, H. S., Greenspan, R. J., and Sokolowski, M. B. (1997). Natural behavior polymorphism due to a cGMP protein kinase of *Drosophila*. *Science* 277:834–836.
O'Tousa, J. E., Leonard, D. S., and Pak, W. L. (1989). Morphological defects in Orajk84 photoreceptors caused by mutation in R1-6 opsin gene of *Drosophila*. *J. Neurogenet.* 6:41–52.
Pak, W. L., Grossfield, J., and Arnold, K. S. (1970). Mutants of the visual pathway of *Drosophila melanogaster*. *Nature* 227:518–520.
Park, Y., Caldwell, M. C., and Datta, S. (1997). Mutation of the central nervous system neuroblast proliferation repressor ana leads to defects in larval olfactory behavior. *J. Neurobiol.* 33:199–211.
Pearn, M. T., Randall, L. L., Shortridge, R. D., Burg, M. G., and Pak, W. L. (1996). Molecular, biochemical, and electrophysiological characterization of. *J. Biol. Chem.* 271:4937–4945.
Pollock, J. A., and Benzer, S. (1988). Transcript localization of four opsin genes in the three visual organs of *Drosophila;* RH2 is ocellus specific. *Nature* 333:779–782.
Porter, J. A., Hicks, J. L., Williams, D. S., and Montell, C. (1992). Differential localizations of and requirements for the two *Drosophila ninaC* kinase/myosins in photoreceptor cells. *J. Cell. Biol.* 116:683–693.
Porter, J. A., and Montell, C. (1993). Distinct roles of the *Drosophila ninaC* kinase and myosin domains revealed by systematic mutagenesis. *J. Cell. Biol.* 122:601–612.
Ranganathan, R., Malicki, D. M., and Zuker, C. S. (1995). Signal transduction in *Drosophila* photoreceptors. *Annu. Rev. Neurosci.* 18:283–317.
Sawin-McCormack, E., Sokolowski, M. B., and Campos, A. R. (1995). Characterization and genetic analysis of *Drosophila melanogaster* photobehavior during larval development. *J. Neurogenetics* 10:119–135.
Schmucker, D., Jackle, H., and Gaul, U. (1997). Genetic analysis of the larval optic nerve projection in *Drosophila*. *Development* 124:937–948.
Sehgal, A., Price, J., and Young, W. (1992). Ontogeny of a biological clock in *Drosophila melanogaster*. *PNAS* 89:1423–1427.
Serikaku, M. A., and O'Tousa, J. E. (1994). Sine oculis is a homeobox gene required for *Drosophila* visual system development. *Genetics* 138:1137–1150.
Steller, H., Fischbach, K. F., and Rubin, G. (1987). *Disconnected:* A locus required for neuronal pathway function in the visual system of *Drosophila*. *Cell* 50:1139–1153.
Tully, T., Cambiazo, V., and Kruse, L. (1994). Memory through metamorphosis in normal and mutant *Drosophila*. J. Neurosci. 14:68–74.
Zipursky, S. L., Venkatesh, T. R., Teplow, D. B., and Benzer, S. (1984). Neuronal development in the *Drosophila* retina: monoclonal antibodies as molecular probes. *Cell* 36:15–26.
Zuker, C. S., Cowman, A. F., and Rubin, G. M. (1985). Isolation and structure of a rhodopsin gene from D. *melanogaster*. *Cell* 40:851–858.

Preface

In the following Chapter, Dr. Ana Regina Campos took the photographs of Wild type, Rh5, and Rh6 Ablated Strains stained with m24B10. The automated tracking system and computer programs written, which facilitated the analysis of larval modulation of locomotion measured by R.I. was set up by Ph.D student, Balaji Iyengar.

Chapter IV: Targeted cell ablations, as a means towards identifying photoreceptor cell-types mediating *Drosophila melanogaster* larval response to light

Introduction:

A behavioural system can be defined in terms of its neural circuit. The role of specific neurons in this circuit can be studied by creating lesions and subsequently assaying for behaviour. For this reason, genetic lesions such as mutations and cell ablations are invaluable towards understanding individual neural function in a behavioural system. In *Drosophila*'s adult visual system, mutations rendering specific photoreceptor cell-types non-functional have assisted in identifying their roles in phototaxis (Benzer *et al.*, 1967; Hotta and Benzer, 1970; Heisenberg and Buchner, 1977; Hu *et al.*, 1980; Miller *et al.*, 1981). Adult photoreceptor cells are classified into three types R1-R6, R7 and R8 based on spectral sensitivities, rhabdomeric position and projection pattern (reviewed by Smith *et al.*, 1991). R1-R6 cells, which express the major blue absorbing rhodopsin, Rh1 (O'Tousa et al., 1995) mediate the optomotor response along with fast phototaxis in dim light (Heisenberg and Buchner, 1977; Miller *et al.*, 1981). The ultraviolet-sensitive R7 cells express Rh3 and Rh4 (Montell *et al.*, 1987; Zuker *et al.*, 1987) while

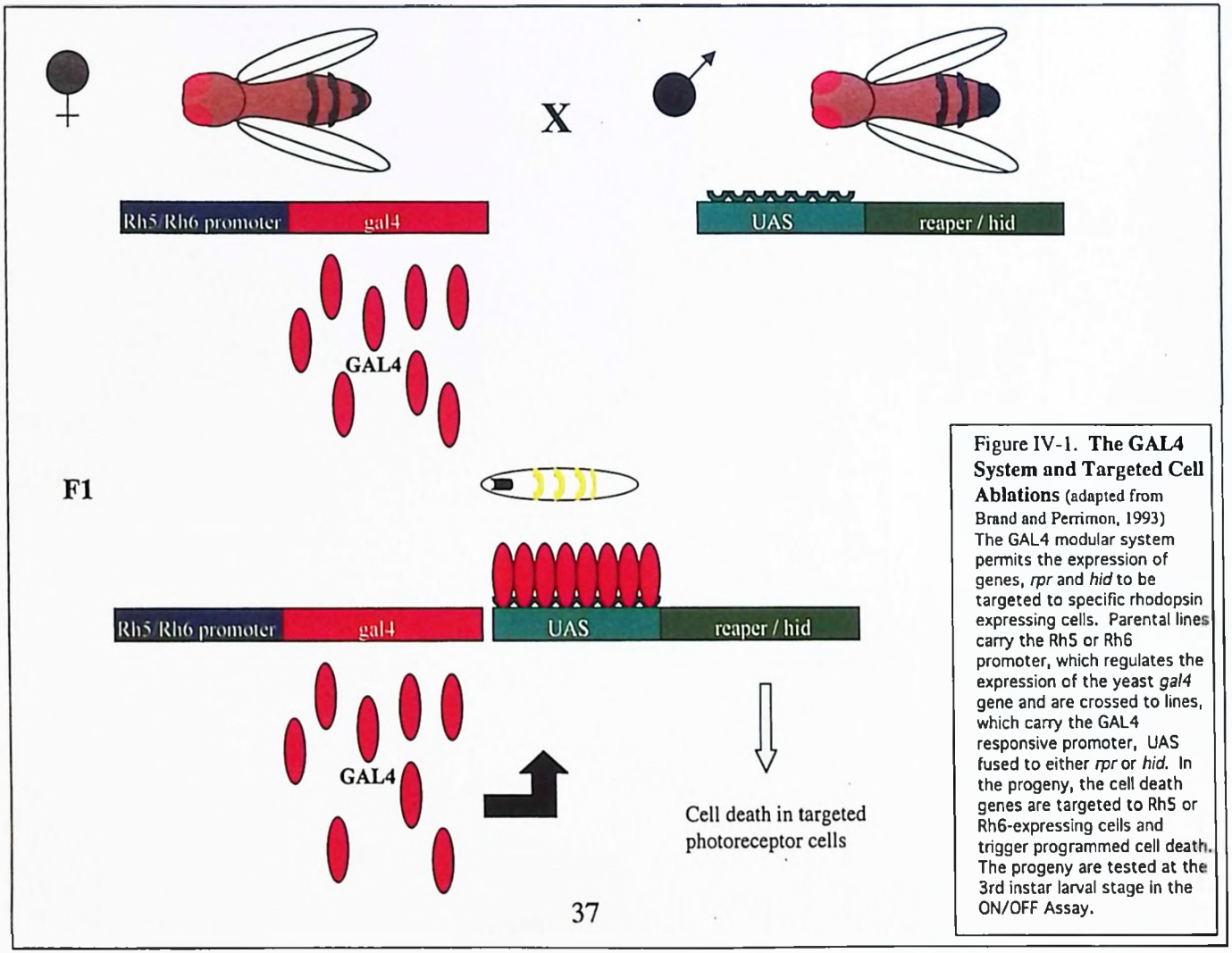

Figure IV-1. **The GAL4 System and Targeted Cell Ablations** (adapted from Brand and Perrimon, 1993) The GAL4 modular system permits the expression of genes, *rpr* and *hid* to be targeted to specific rhodopsin expressing cells. Parental lines carry the Rh5 or Rh6 promoter, which regulates the expression of the yeast *gal4* gene and are crossed to lines, which carry the GAL4 responsive promoter, UAS fused to either *rpr* or *hid*. In the progeny, the cell death genes are targeted to Rh5 or Rh6-expressing cells and trigger programmed cell death. The progeny are tested at the 3rd instar larval stage in the ON/OFF Assay.

Table IV-I Spectral Sensitivities and Expression patterns of opsin genes in Drosophila melanogaster (reviewed by Montell, 1999)

Rhodospsin	Expression	Absorption
Rh1	Larval ? and Adult	480
Rh2	Adult	420
Rh3	Adult	345
Rh4	Adult	375
Rh5	Larval and Adult	440
Rh6	Larval and Adult	510

R8 cells express blue-absorbing Rh5 and (Papatsenko *et al.*, 1997) green-absorbing Rh6 in non-overlapping subsets of cells (Chou *et al.*, 1996;1999). Both R7 and R8 cells mediate fast phototaxis in high intensity light along with some aspects of slow phototaxis (Heisenberg and Buchner, 1977).

Cell ablations in combination with behavioural analyses offer a means to uncover function of subsets of neurons. Selective cell ablations can be accomplished through a variety of techniques. Physical ablation of cells through the use of UV microbeam lasers has been undertaken to selectively ablate neurons in *C. Elegans* (Avery *et al.*, 1987). The inaccessibility of cells to be ablated along with the invasiveness of this type of manipulation are obstacles in conducting physical ablations, although in *C. Elegans,* non-invasive ablations have been conducted (Avery *et al.*, 1987). The vertebrate zebrafish, has emerged as a promising model to study the cellular basis of behaviour. Recently, the escape response has been analyzed by taking advantage of the transparent larval stage to carry out non-invasive photoablations (reviewed by Fetcho and Liu, 1998). The aforementioned problems associated with physical ablations may be circumvented through the use of genetic ablations. In *Drosophila*, cell ablations have been attempted through toxigenic approaches. Temperature-dependent ablations of specific cells was accomplished through selective expression of toxins

ricinA chain and diphtheria toxins directed by characterized enhancers (Kunes and Steller, 1991; Moffat *et al.*, 1992).

Subsequent development of the *GAL4* system, provided a new method of genetic cell ablation through which toxin expression could be targeted to specific cell-types (Brand and Perrimon, 1993). The *GAL4* system permits two viable transgenic lines one, carrying the toxin gene or the target gene another the characterized promoter fused to *gal4* gene (Figure IV-1). The target gene is only activated in the progeny, where both GAL4 and the *GAL4*-responsive promoter, UAS are present. This system has also been used to express tetanus toxin light chain which results in the elimination of synaptic transmission in targeted neurons in the nervous system and neuromuscular junction (Sweeney *et al.*, 1994). Similarly, the p[UAS]/p[Gal4] system can be employed such that under the direction of a cell specific promoter the cell death genes, *head involution defective* (*hid*) (Grether *et al.*, 1995) and *reaper* (*rpr*) (White *et al.*, 1988) can be expressed to target and obliterate specific neurons. The latter strategy was first implemented to target neuropeptide involvement in complex behavioural programs such as circadian rhythm behaviour and also to gain insight into the development of central nervous system (McNabb *et al.*, 1997; Zhou *et al.*, 1997; Renn *et al.*, 1999).

Busto *et al.*, 1999 and Hassan *et al.*, 2000 employed two novel photobehaviour assays in an effort to genetically dissect aspects of larval light response: the ON/OFF assay and the Checker assay. Both assays unequivocally support the notion that other rhodopsins are capable of mediating aspects of the response to light (Busto *et al.*, 1999; Hassan *et al.*, 1999). Confirmation of the expression of rhodopsins Rh1, Rh5 and Rh6 in the Bolwig's organ has been sought through the use of promoter fusions and immunohistochemical analysis (C. Desplan, personal communication). However, Rh3 and Rh4 are not expressed as previously reported in Pollock and Benzer 1988 (C. Desplan, personal communication). This leaves the question open as to which photoreceptor cell-types mediate aspects of larval light response in our assays. In order to address this question genetic cell ablation presents itself as an enticing means towards studying the role of individual neurons and larval photoreceptor cell types in photobehaviour.

Here, I report the use of the p[UAS]/p[Gal4] system to target *rpr* and *hid* to photoreceptor cells expressing Rh5 and Rh6. The ensuing analysis involved assaying the Rh5 and Rh6 ablated strains for larval photoresponse in the ON/OFF Assay (Busto *et al.*, 1999). The developmental consequences of ablation were assessed immunohistochemically, using photoreceptor-specific monoclonal antibody m24B10 and X-gal staining to assess the degree of ablation.

Chapter IV: Results

Ablation of Rh5 but not of Rh6 -expressing Cells Disrupts the Modulation of Locomotion by Light Measured in the ON/OFF Assay

In order to determine the role of specific rhodopsin expressing cells in the various responses to light, larvae in which these photoreceptors were missing were tested in the ON/OFF assay. Ablation of these cells was accomplished using the *GAL4* modular system to target the expression of the cell death genes *hid* or *rpr*. Lines carrying the yeast *gal4* gene under the regulation of the Rh5 or Rh6 promoter (Papatsenko *et al.*, 1997; Huber *et al.*, 1997) were crossed to lines carrying either cell death genes under the *GAL4*-responsive promoter UAS. The expression of either cell death gene is sufficient to trigger programmed cell death in larval and adult photoreceptors (Renn *et al.*, 1997; McNabb *et al.*, 1999; Busto *et al.*, 1999; Hassan, *et al.*, 2000). The larval progeny of these crosses were tested in the ON/OFF assay, which allows for the assessment of the three different responses to light. These respnses are the reduction in the path length during the light, increased frequency in head swinging in the light and angle of direction changes at various light transitions.

An automated tracking system facilitated the analysis of larval modulation in locomotion, permitting the simultaneous testing and distance tracking of larvae (B. Iyengar, Ph.D thesis and unpublished results). A Response Index (R.I) was calculated and takes into account the relative distances the larva moved in light versus dark. Head swinging behaviour and angle of direction change in larval paths were analyzed after manually tracing larval paths recorded on videotape. The NIH image analysis software downloaded from public domain facilitated the analysis of the angle measurements (see Busto M.Sc. materials and methods for details and Busto *et al.*, 1999).

On average, third instar 'foraging' wild type larvae between the ages of 84 and 90 hr. (AEL) tend to travel a greater distance in the dark versus light pulses (Busto *et al.*, 1999; Fig. IV-2, Fig. IV-3). The reduction in path length observed during light pulses may be explained by an increase in the frequency of larval stops and head swings in the light and/or a direct reduction in locomotory speed. A response index is derived (R.I.=d(Dark)-d(Light)/d(Total)) which takes into account the distance traveled in the dark pulses versus light pulses relative to the total distance moved in the assay.

Both wild type strains *OregonR* and *CantonS* exhibit a robust response to light as reflected in a R.I. of around 0.3 (Fig. IV-2 and Fig. IV-3). This value reflects a reduction in path length of about 50% in the light versus the dark.

In the parental lines carrying the *GAL4*-responsive under the UAS promoter, the cell death gene is silent. In the parental lines carrying the yeast *gal4* gene under the promoter of Rh5 or Rh6, GAL4 is expressed, but is unable to activate in the absence of a target.

Parental Strains Rh5-*gal4* (ANOVA: Rh5-*gal4*, $F_{1,29}$=16.43, p=0.001; $F_{1,14}$=50.05, p<0.001), *UAS-hid* (ANOVA: UAS-*hid*, $F_{1,29}$=15.85, p=0.007) and *UAS-rpr* (ANOVA: UAS-*rpr*, $F_{1,29}$=16.43, p=0.001) were assayed and their responses were not significantly different from ablated strains Rh5-*gal4*xUAS-*rpr* and Rh5-*gal4*xUAS-*hid*, respectively (Fig. IV-2 and Fig. IV-3). A significant reduction in the R.I. was observed only in the larval progeny carrying Rh5-*gal4* as well as either cell death gene under the control of the *GAL4*-responsive promoter, UAS as compared to wild type *OregonR* and *CantonS*, respectively (ANOVA: *Rh5-gal4xUAS-rpr*, $F_{1,29}$=50.05, p<0.001, $F_{1,29}$=26.76, p<0.001), and (ANOVA: *Rh5-gal4xUAS-hid*, $F_{1,19}$=53.23, p<0.001, $F_{1,19}$=53.23, p<0.001)(Fig. IV-2).

Larvae in which the cell death gene, *rpr* was targeted to Rh6-expressing cells displayed a mean R.I. just under 0.2 (Fig. IV-3), which means that on average the larvae reduce their path length by about 35% in the dark compared to light. There is however, a statistical difference between the Rh6-*gal4*xUAS-*rpr* strain and both wild type strains (ANOVA: Rh6-*gal4*xUAS-*rpr*,

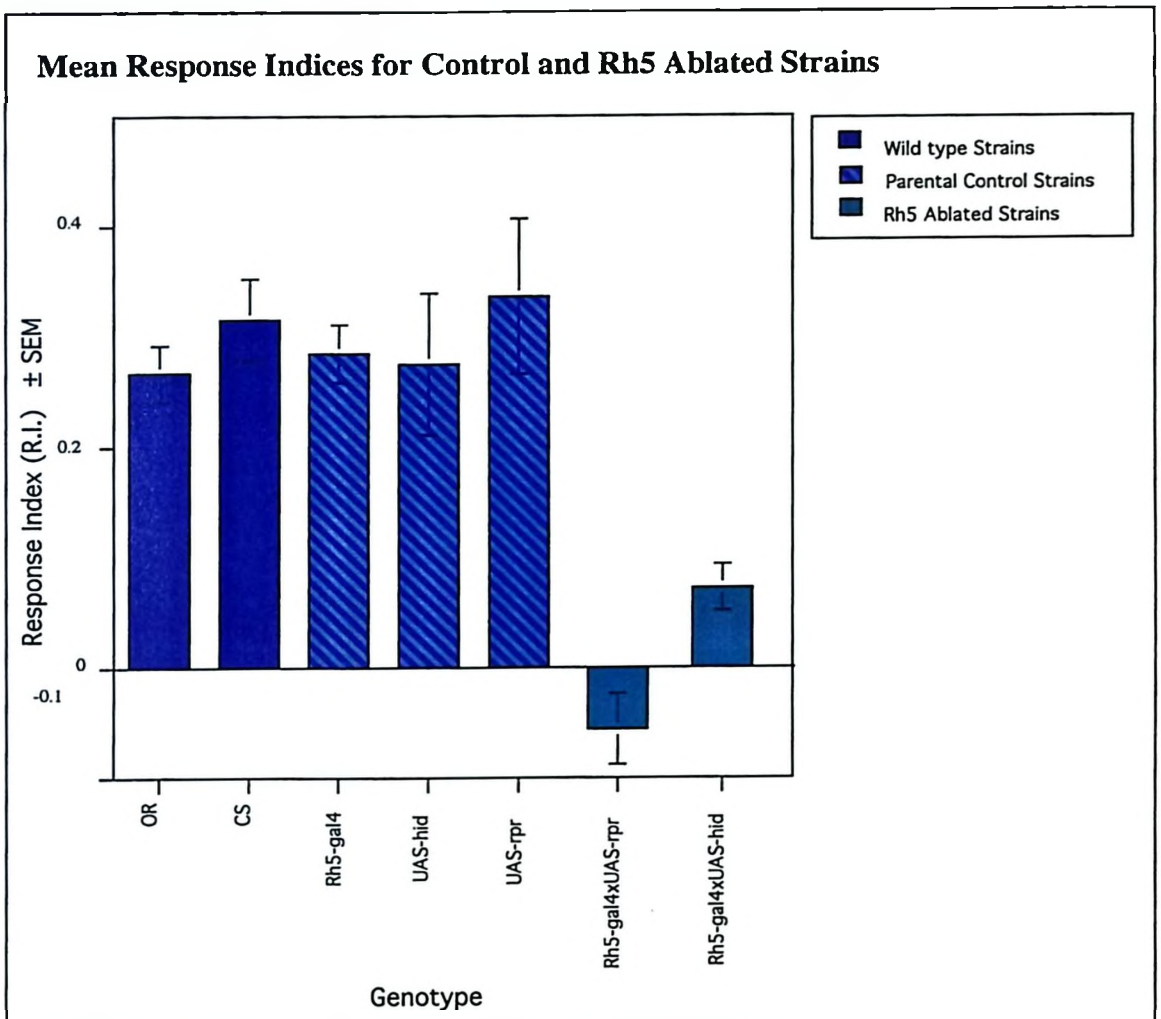

Figure IV-2. Mean Response Indices (R.I.'s) for Control and Rh5 Ablated Strains
The increase in path length in the light as seen in wild type strains OregonR (n=20) and CantonS (n=20) is abolished in both strains in which Rh5 cells been ablated by cell death genes, Rh5-gal4xUAS-rpr (n=30) $p<0.001$ and Rh5-gal4xUAS-hid (n=20) $p<0.001$ ($p<0.001$ for all comparisons with wild type CS and OR). When R.I.'s for Rh5 ablated strains are compared to parental strains, the same trend is found. Responses of Rh5-gal4xUAS-rpr and Rh5-gal4xUAS-hid are significantly different from parental strain Rh5-gal4 (n=15) $p=0.001$ and $p<0.001$, respectively. Parental strains UAS-hid (n=7) and UAS-rpr (n=7), are also significantly different from ablated strains $p=0.004$ and $p=0.007$, respectively.

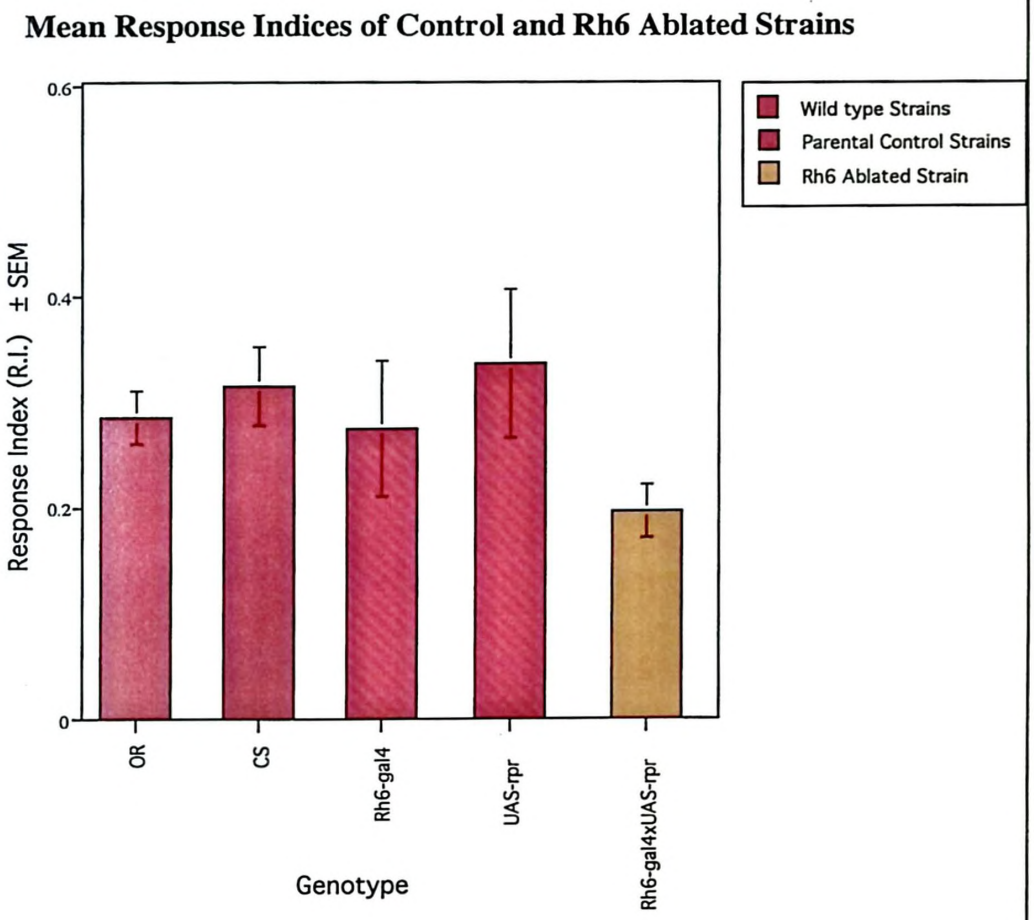

Figure IV-3. Mean Response Indices (R.I's) of Control and Rh6 Ablated Strains
The increase in path length in the light as seen in wild type strains OregonR (n=20) and CantonS (n=20) is reduced in the strain in which Rh6 cells have been ablated by cell death gene, Rh6-gal4xUAS-rpr (n=22) p=0.033, p=0.008 for comparisions with both wild type strains OregonR (n=20) and CantonS (n=20), respectively. When R.I.'s for Rh6 ablated strains are compared to parental strains, the response is not significantly different Rh6- gal4 (n=6) and UAS- rpr (n=7) p=0.164 and p=0.099, respectively, from the ablated strain, Rh6- gal4x UAS- rpr (n=22). Sample sizes for the parental strains are small and should be increased.

$F_{1,21}$=8.93, p=0.008, $F_{1,21}$=5.25, p<0.001, p=0.033) compared to *OregonR* and *CantonS*, respectively (Fig. IV-3). No significant statistical difference was calculated between parental strains and Rh6-ablated strain and two parental strains, Rh6-*gal4* (ANOVA: Rh6-*gal4*, $F_{1,21}$=2.65, p=0.164) and *UAS-rpr*, (ANOVA: UAS-*rpr*, $F_{1,21}$=4.56, p=0.099), respectively (Fig. IV-3).

These results indicate that Rh6-*gal4*xUAS-*rpr* displays a reduction in path length in the light, however this response is reduced in comparison to wild type. The degree of reduction is quite different from Rh5-ablated strains.

Ablation of Rh5 but not of Rh6-expressing Cells Disrupts the Increase in Head Swinging Behaviour Triggered by Light Measured in the ON/OFF

When wild type larvae were subjected to 10s light and dark pulses in the ON/OFF assay, visual inspection revealed that larvae perform a characteristic head swinging behaviour in response to light. Quantitative analysis revealed that during the light pulse wild type larvae perform this behaviour at a greater frequency, than during dark pulses (Busto *et al.*, 1999; Fig. IV-4 and Fig. IV-5 (ANOVA: *CantonS*, $F_{1,19}$=55.84, p<0.001) (ANOVA: *OregonR*, $F_{1,18}$=108.41, p<0.001). A comparison between light and dark conditions revealed that parental strains also displayed an increased number of mean head swings per light pulse than per dark (ANOVA: Rh5-*gal4*,

$F_{1,4}$=45.61, p<0.003), (ANOVA: UAS-*hid*, $F_{1,8}$=9.08, p=0.017) and (ANOVA: UAS-*rpr*, $F_{1,4}$=7.86, p=0.029) (Fig. IV-4). Head swinging behaviour was abolished in Rh5-ablated strains, (ANOVA: *Rh5-gal4*x*UAS-rpr*, $F_{1,21}$=0.65, p=0.430) and (ANOVA: *Rh5-gal4*x*UAS-hid*, $F_{1,18}$=0.27, p=0.610) (Fig. IV-4) but not in the Rh6 ablated strain (ANOVA: *Rh6-gal4*x*UAS-rpr* $F_{1,23}$=18.0, p=0.001) (Fig. IV-5).

Taken together, the R.I's and head swinging behaviour analyses support the notion that the blue-absorbing Rh5 is necessary in the mediation of these two aspects of the response to light. As for the *Rh6-gal4* x*UAS-rpr*, the results are less definite. In the event of a non-response, theoretically the larvae should travel an equal distance during dark pulses and light pulses, which translates into a response index of 0. The measured mean R.I. of 0.2 suggests the larvae are responding, not quite to the same degree as the wild type strains, but still well above a non-responding strain (Fig. IV-5). Furthermore, the head swinging data indicates that the Rh6-ablated strain is able to detect light.

When Busto *et al.*, (1999) tested a variety of mutants in genes known to function in the adult phototransduction cascade, response to light as measured through R.I. and head swinging behaviour appeared correlated with each other. Larvae, which displayed a mean response index similar to that of wild type also displayed increased head swinging in the light (Busto *et al.*,

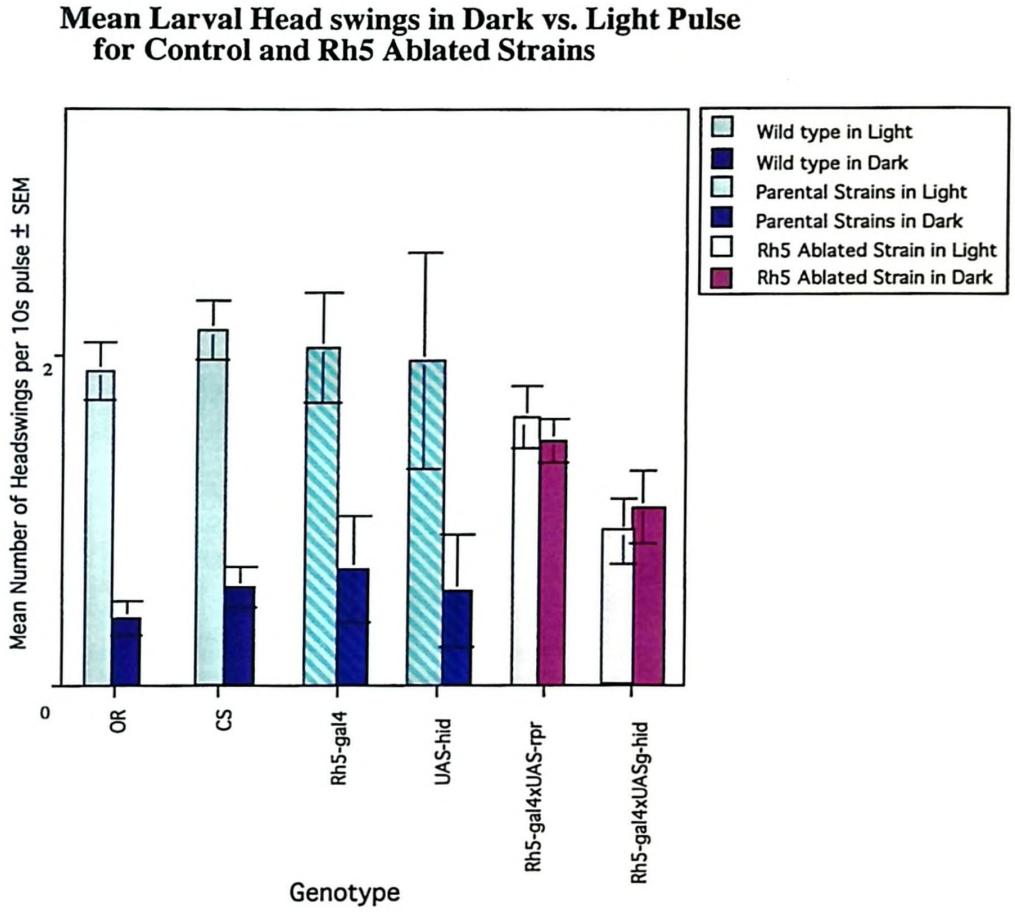

Figure IV-4. Mean Larval Head swings in Dark vs. Light Pulse for Control and Rh5 Ablated Strains
Both wild type strains OregonR (n=19) and CantonS (n=20) display an increased frequency of head swinging in the light vs. the dark. A significant difference in mean number of headswings per light pulse vs. dark pulse was calculated $p<0.001$, for both wild type strains. In both R5 ablated strains, however the increased frequency of head swinging in the light is abolished, Rh5-gal4xUAS-rpr n=22 p=0.430 and Rh5-gal4xUAS-hid n=19 p=0.610. The parental control strains display an increased head swinging in the light, Rh5-gal4 (n=15) $p<0.001$, UAS-hid (n=9) p=0.017 and UAS-rpr (n=5) p=0.029.

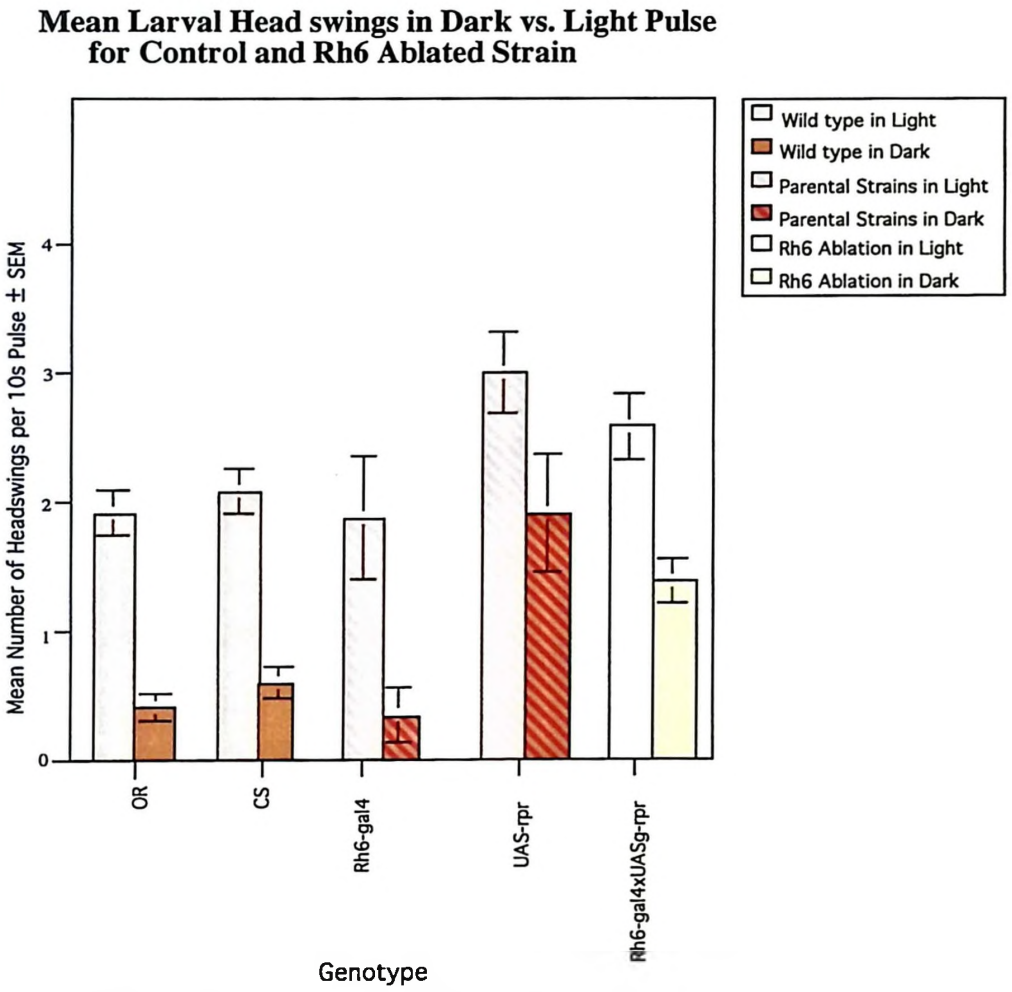

Figure IV-5. Mean Larval Head swings in Light vs. Dark Pulses for Control and Rh6 Ablated Strains
Both wild type strains OregonR (n=19) and CantonS (n=20) display an increased frequency of head swinging in light vs. the dark pulses. A significant difference in mean number of headswings per light pulse vs. dark pulse was calculated $p<0.001$, for both strains. In the Rh6 ablated strain, an increased frequency of head swinging in the light is also present, Rh6-gal4xUAS-rpr n=22 $p<0.001$. The parental control strains display an increased head swinging in the light, Rh6-gal4 (n=6) $p<0.001$, and UAS-rpr (n=7) $p=0.029$, however sample sizes should be increased.

1999). This led to the conclusion that the genetic cascade mediating both larval photobehaviours was based on an adult-like phototransduction cascade. Neither behaviours are abolished in *ninaE* mutants, which suggests that other rhodopsins may mediate the response to light as measured by R.I. and head swinging behaviours. It appears these behaviours known to be mediated via an adult-like phototransduction cascade are mediated via Rh5.

Response to Light as Measured by Changes in Magnitude of Angle of Direction Change in Larval Path at Light Transitions not Interpretable

The angle of direction change in wild type larval path at light transitions decreases in magnitude in the following order, Dark to Light (DL), Light to Dark (LD) and Dark to Dark (DD). Wild type controls are able to distinguish between a complete absence of light versus lights being turned on or off (Fig. IV-6 and Fig. IV-7; Busto *et al.*, 1999). Parental control strains, however are all unable to distinguish between any of the light transitions Rh5-*gal4* (ANOVA: *Rh5-gal4* $F_{2,18}$=021, p=0.811), Rh6-*gal4* (ANOVA: Rh6-*gal4*, $F_{2,5}$=3.35, p=0.082, UAS-*hid* (ANOVA: UAS-*hid*, $F_{2,6}$=0.84, p=0.458) and UAS-*rpr* (ANOVA: UAS-*rpr*, $F_{2,5}$=0.27, p=0.770)(Fig. IV-6 and Fig. IV-7). Therefore, the effect of Rh5 and Rh6 photoreceptor ablation on this behaviour cannot be evaluated. Increasing the sample sizes of the parental control strains might yield different results and should be undertaken. If the result

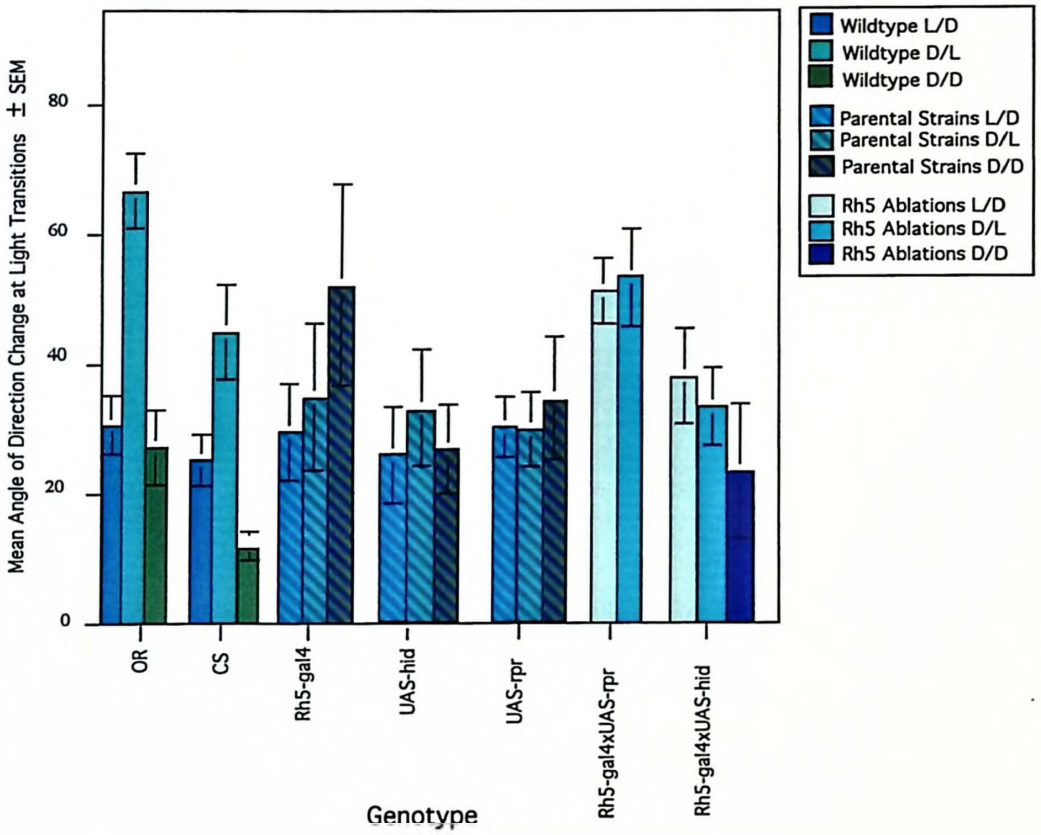

Figure IV-6. Mean Angle of Direction Change at Light Transitions for Controls and Rh5 Ablated Strains

Angle of direction change at light transitions for wild type strain CantonS (n=18) is significantly different ($p<0.001$) between all light transitions. Other wild type strain, OregonR (n=18), also shows a difference between light transitions ($p<0.001$), however this difference between the light to dark (L/D) and absence of light (D/D) transitions is not statistically significant. Parental Controls Rh5-gal4, UAS-hid, and UAS-rpr are all not significantly different at light transitions, based on smaller sample sizes for the first two parental strains, $p=0.770$, $p=0.458$ and $p=0.811$, respectively. In the Rh5 ablated strains, Rh5-gal4xUASg-rpr n=24, $p=0.769$ and Rh5-gal4xUASg-hid n=16, $p=0.899$ there is no significant difference between any of the light transitions.

is still the same, then the genetic background of the strains should be changed.

Integrity of Remaining Cells not Compromised in Rh5 Ablated Strains

The targeted ablation of photoreceptor cell-types raises two main concerns namely, the non-cell autonomous effect of ablation and the extent of ablation. More specifically, a lack of response of the Rh5-ablated strains may be attributed to disruption in the development of the non-ablated remaining cells (Rh6 expressing cells). Immunohistochemistry was employed in order to assess the integrity of the remaining photoreceptor cells. The primary monoclonal antibody MAb24B10 was used in conjunction with HRP (HorseRadish Peroxidase). MAb24B10 recognizes CHAOPTIN, a 160 kd glycoprotein expressed specifically on the photoreceptor-cell plasma membrane (Van Vactor, 1988). Third instar larvae in which Rh5-expressing cells have been ablated show larval as well as adult axonal projection patterns that are indistinguishable from wild type (Fig. IV-8 and Fig. IV-9). The Bolwig's nerve (BN) is present in Rh5-*gal4* xUAS-*rpr* and Rh5-*gal4* xUAS-*hid* strains and projects ventrally and posteriorly with its terminus innervating the brain hemisphere (Fig. IV-8 and Fig. IV-9). Similarly, adult projections terminate in the medulla and R7 and R8 axons project through the medulla terminating in the lamina (Fig. IV-9).

There appears to be no indication that the integrity of the remaining photoreceptor cells is compromised. The lack of response seen in larvae expressing *hid* or *rpr* in the Rh5 photoreceptors is the result of the cell-specific ablation, as opposed to the developmental disruption of remaining photoreceptor cells.

When Rh6-*gal4* xUA*S-rpr* and Rh6-*gal4* xUA*S-hid* strains were stained with MAb24B10 the projection of BN, but not the adult photoreceptor cell axons appears affected (Fig. IV-9). In all *Rh6-gal4* x*UAS-rpr* larvae no BN was visualized whatsoever, whereas in a few of the *Rh6-gal4* x*UAS-hid* (data not shown) a very faint BN could be detected under the microscope. Promoter fusions indicates that Rh6 is expressed in 7-8 of the 12 photoreceptor cells and Rh5 is expressed in only 3-4 cells (C. Desplan, personal communication). It is possible that remaining axons are below the level of detection in our specimens. The question regarding the developmental consequences of Rh6 cell ablation remains open and should be investigated at a higher resolution.

Extent of Ablation Imparted by hid Under Control of Rh5 Promoter Appears to be Complete

In order to assess the degree of ablation, the Rh5-ablated strain, Rh5-*gal4x*UAS-*hid* was crossed into a UAS-*tau-lacZ* background and stained with

X-gal. In this background the *lacZ* reporter gene is driven in all Rh5 expressing cells which have not been successfully ablated on the part of the cell death gene, *hid*. Non-ablated cells can be visualized through the presence of deep blue staining.

Third instar larval brains were dissected from the Rh5-*gal4*xUAS-*hid*x*UAS-tau-lacZ* strain and double-labeled with X-gal and mAb24B10 (see Appendix C., Cross Scheme). Brains displayed no blue X-gal staining and subsequent staining with photoreceptor cell specific mAb24B10 suggests that the integrity of the remaining photoreceptor cells was not compromised (Fig. IV-11). The control *glass-lacZ* in which the reporter gene is directly fused to the *gl* promoter was similarly double-labeled and exhibited blue staining of larval photoreceptor cells (Fig. IV-11). This suggests that all Rh5-expressing cells have been successfully ablated, however such a conclusion must be tentative in light of the absence of other appropriate controls (see Discussion).

Targeted Ablation of Subsets of Photoreceptor Cells Does not Impair General Locomotion

The response to light as measured by R.I. depends upon the capacity of the larvae to move in the ON/OFF. Measuring the mean distances traveled in 30s in complete darkness assisted in the assessment of the locomotory ability of each of the strains tested in the ON/OFF Assay. Parental Strains UAS-*hid*,

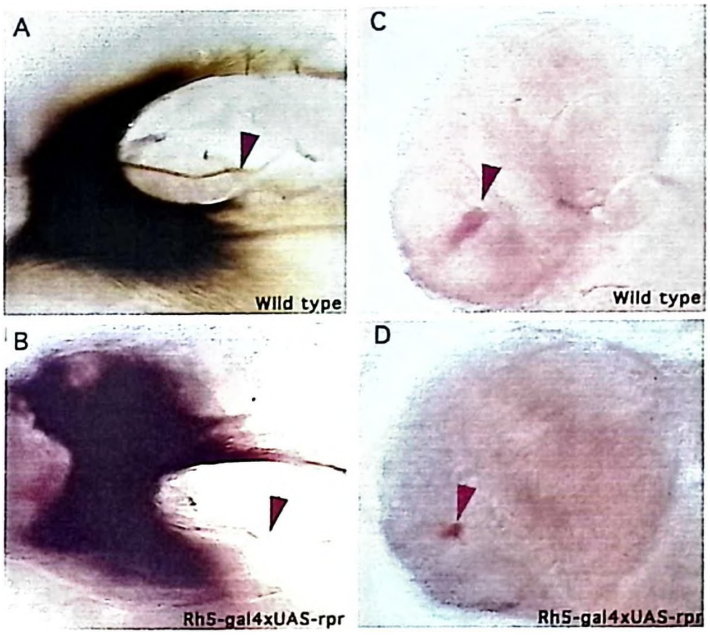

Figure IV-8. Larval Optic Nerve projects to and terminates in Optic Lobe Primordium in Wild type and Rh5 Ablated Strains

In young wild type third instar larvae, the photoreceptor cells are located in bilateral clusters in a dorsal epithelial pouch near the mouth hooks. Young larval brains were stained using photoreceptor-specific monoclonal antibody m24B10. The cells extend their axons, which fasciculate into Bolwig's Nerve (BN) in both wild type, Rh5-gal4xUAS-rpr and Rh5-gal4xUAS-hid (Arrow). The BN innervates the optic lobe primordium, and the terminus can be visualized in wild type as well as both wild type and Rh5-ablated strains (Panels B and D, Arrows). The BN is completely absent in Rh6-gal4xUAS-rpr strain (data not shown).

Figure IV-9. Late Third Instar Larvae in which Rh5 Cells have been Ablated have Wild type Adult and Larval Projections, Rh6 Ablated Strain only has Normal Adult Projections
In wild type strain (n=25) the larval optic nerve projects and terminates in the brain hemisphere (Red Arrow). Also, adult photoreceptors differentiate in the eye imaginal disk (R1-R6) and project their axons to the first optic ganglia (lamina) (Blue Arrow) and (R7 and R8) to the second optic ganglia (medulla) (Purple Arrow). Rh5-gal4xUAS-rpr(n=25) and Rh5-gal4xUAS-hid (n=25) show adult photoreceptor projections (Blue and Purple Arrows), as well as larval optic nerve projections (Red Arrow). Both of these projections appear no different than wild type. In Rh6-gal4xUAS-rpr (n=20), the adult photoreceptor projections are as in wild type specimens, however the larval optic nerve is absent (Red Arrow, Panel C).

Locomotion of Control and Specific Rhodopsin Ablated Strains

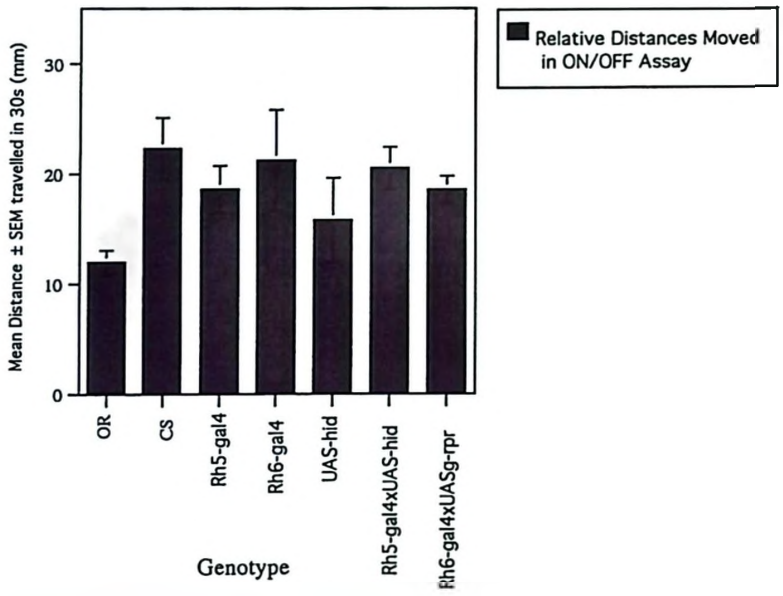

Figure IV-10. Locomotion of Control and Specific Rhodopsin Ablated Strains
Rh5-gal4 (n=17) p=0.01, p=0.617, UASg-rpr (n=7) p=0.008, p=0.806 and UAS-hid (n=8) p=0.045, p=0.591 move a greater distance in 30s and are significantly different from Oregon R (n = 24) but not from CantonS (n=17), respectively. Distance moved in 30s for Rh6-gal4 (n=4) p=0.083, p=0.891 is not significantly different from OregonR (n=24) or CantonS, respectively. Both ablated strains Rh5-gal4xUASg-hid (n=19) p=0.001, p=0.829 and Rh6-gal4xUAS-rpr (n=6), p=0.013, p=0.992 move a distances in 30s that is significantly different from OregonR, but mean distances are significantly greater than this wild type strain (second p-value shows they are not different from CantonS). Both wild type strains are significantly different from each other.

Figure IV-11. Degree of Ablation appears complete in Rh5-ablated strain
Control gl-lacZ (n=5) and rh5-gal4xUAS-hidxUAS-tau-lacZ are double-labeled with X-gal and mAb24B10 (n=5). The control shows blue staining of larval optic nerve projection (Panel A) and adult projections (Panels A and B, Arrows). In Rh5-gal4xUAS-hidxtau-lacZ, no blue staining is present (Panel C) suggesting all rh5 cells have been ablated, and the integrity of the remaining photoreceptor cells is not compromised.

(ANOVA, UAS-*hid*, $F_{1,21}$=0.5.97, p=0.045, $F_{1,16}$=0.32, p=0.591), UAS-*rpr*, (ANOVA, UAS-*rpr*, $F_{1,23}$=15.14, p=0.008, $F_{1,16}$=0.07, p=0.806), Rh5-*gal4* (ANOVA, Rh5-*gal4*, $F_{1,23}$=8.40, p=0.010, $F_{1,16}$=0.26, p=0.617) and Rh6-*gal4* (ANOVA, Rh6-*gal4*, $F_{1,23}$=6.60, p=0.083, $F_{1,16}$=0.02, p=0.891) were compared to *CantonS* and *OregonR*, respectively. As well, Rh5 and Rh6 ablated strains were compared to wild type and showed no evidence of a locomotory deficit . Both were significantly higher than *OregonR*, (ANOVA, Rh5-*gal4*xUAS-*hid*, p=0.001), (ANOVA: Rh6-*gal4*xUAS-*hid*, $F_{1,23}$=13.95, p=0.013) (Fig. IV-10). In fact, all strains displayed locomotory ability on par with *CantonS*, which moved a mean distance of about 25 mm in 30s. The locomotory ability of these strains was significantly greater than *OregonR* indicating that movement in the assay is well above the threshold necessary for the behavioural response tested. Hence, lack of locomotion does not appear to be a confounding factor in the analysis.

Chapter IV: Discussion

Rh5 mediates Response to Light in Larval Photoreceptor Neurons, via Pathway Genetically Similar to Adult Phototransduction Cascade

When wild type *Drosophila* larvae are tested in the ON/OFF Assay and exposed to intermittent 10s pulses of light and dark, the larvae qualitatively reveal characteristic behaviours. Wild type larvae characteristically stop and swing their heads from side to side during the light pulses, whereas during the dark pulses, they take a much straighter path. Furthermore, when the lights are turned on the larvae tend to make sharp changes in path direction.

The ON/OFF Assay facilitates the quantification of three discrete aspects of behaviour. In Rh5-ablated strains, Rh5-*gal4*xUAS-*rpr* and Rh5-*gal4*xUAS-*hid* two of the behaviours typically seen in wild type larvae are abolished: the increased frequency in head swinging and the decreased path length during the light pulse. These same two behaviours have been shown to be disrupted by mutations in genes operating in the adult phototransduction cascade and mediated in photoreceptor cells, which differentiate under the control of both transcription factors, *so* and *gl* (Busto et al., 1999). Thus, it appears that the blue-absorbing rhodopsin, Rh5 mediates response to light via a pathway whose components are similar to those found in the adult phototransduction cascade.

Furthermore, secondary issues arising from the ablation experiments were addressed. Staining with monoclonal antibody, m24B10 which is specific for CHAOPTIN, and is specifically expressed in photoreceptor cell bodies and axons, showed that the projection pattern of the remaining photoreceptor cells in the ablated strains are unaffected (Van Vactor et al., 1988). Thus, a lack of response cannot be attributed to the altered projection patterns of remaining photoreceptor cells and appears to be a result of the ablation itself. Third instar Rh5-*gal4*xUAS-*hid* larval brains, double-labeled with m24B10 and X-gal staining, support that all Rh5-expressing cells have been ablated. However, this conclusion is tentative, pending the proper controls (see general discussion).

Independent Genetic Pathway Previously Revealed through Change of Direction Analysis: the Roles of Rh5 and Rh6 Remain Concealed

The sharp change in path at light transitions has been compared in wild type larvae. This analysis has revealed that at light transitions, larvae change the direction in path in the following order of decreasing magnitude: Dark to Light (D/L)> Light to Dark (L/D) > Dark to Dark (D/D) or absence of light (Busto et al., 1999; Fig. IV-6 and Fig. IV-7). Subsets of the angle of direction change behavior are mediated on the part of an independent pathway, since mutations in genes operating in the adult phototransduction cascade do not

abolish these light responses (Busto *et al.*, 1999). The roles of Rh6 and Rh5 in this pathway remain concealed, since the parental strains did not respond as wild type at the light transitions (ie. the magnitude of direction change at the light transitions was abolished). Thus, no conclusions can be inferred from the angle of direction change results obtained for the Rh5 and Rh6-ablated strains. The role of Rh6 remains unclear at this point.

Different Rhodopsins are Known to Possess Unique Spectral Sensitivities: Entertaining the Possibility that Different Aspects of Larval Visual Response are Wavelength-Specific

Promoter fusions have been employed to detect the expression of Rh5 and Rh6 and indicate that Rh5 is expressed in approximately, 3-4 cells, and Rh6 in 7-8 of the remaining cells (C. Desplan, personal communication). From a solely quantitative point of view, there are a greater number of remaining photoreceptor cells to mediate a response in Rh5 ablated strains than Rh6 ablated strains. However, Rh5 and Rh6 possess unique spectral sensitivities, from which it may be surmised that perhaps the behaviours are wavelength-specific. If different aspects of larval visual response are wavelength specific then perhaps insight into the function of Rh6 may be gained through the stimulation of this particular rhodopsin with green-absorbing, 508nm light.

Chapter IV: General Discussion

Mutations and Targeted Ablations help define Molecules and Photoreceptor Cell-types required for Larval Response to Light

The ON/OFF and Checker Assays have facilitated our understanding of the molecular and cellular mechanisms underlying larval response to light. With the goal of untangling the network of molecules and cell-types mediating larval visual response, a genetic strategy was undertaken. This strategy involved testing mutations in genes required for adult phototransduction and visual system development (Busto *et al.*, 1999; Hassan *et al.*, 2000). Cell-specific ablations of Rh5 and Rh6 expressing photoreceptor cells have permitted the identification of cell-types mediating larval response to light. Furthermore, genetic screening, facilitated through our assays, has uncovered a novel visual system mutant (Iyengar *et al.*, 2000). When adult phototransduction cascade mutants are tested in the ON/OFF Assay, two behavioural responses are abolished: reduced path length in the light, increased head swinging behaviour in the light and the capacity to distinguish between D/L and L/D transitions (Busto *et al.*, 1999). These mutations also disrupt the response to light as measured in the Checker

Assay (Hassan *et al.*, 2000). The adult phototransduction cascade mutations which disrupt these light responses include the following genes: *norpA*, which encodes the enzyme PhospholipaseC (PLC) and *trp*, which encodes a calcium ion channel (Busto *et al.*, 1999; Hassan *et al.*, 2000). *NinaC* a gene, which encodes a cytoskeletal protein consisting of a protein kinase domain joined to a myosin heavy chain (Montell and Rubin, 1988), has been shown to be important in mediating the adult phototransduction cascade (Porter *et al.*, 1992; 1993). This gene was reported to also mediate HS and larval path reduction in the ON/OFF assay (Busto *et al.*, 1999), when the *ninaC* null mutant was recently re-tested, these behaviours were unaffected (see Appendix B). These results support the presence of a signaling cascade, similar to the adult phototransduction cascade which mediate HS and path length reduction behaviours in the larva.

Mutations in the *gl* and *so* genes, as well as the targeted expression of cell death genes have been particularly informative in defining the role of cell types in mediating light response (Busto *et al.*, 1999; Hassan *et al.*, 2000). To determine whether photoreceptor cells in the Bolwig's organ (BO) mediate responses to light, as measured in the ON/OFF and Checker Assays, we used mutations which disrupt larval visual system development. The *gl* gene is required in both larval and adult photoreceptor cell differentiation. In the larva, the *gl* gene is also expressed in two groups of cells in the central brain.

The *so* gene is expressed in both the optic lobe primordium and larval photoreceptor cells and is required for proper visual system determination (Serikaku and O'Tousa, 1994). In the Checker assay, a response to light is quantified through the calculation of a response index based on residence time in the dark versus light conditions [R.I. = t(dark)-t(light)/t(dark+light)]. Hence, a response to light is seen as an increased residence time in the dark. This response is abolished in the so^{mda} mutant and *glass multimer reporter-head involution defective* (pGMR-*hid*) strain (Hassan et al., 2000). These results imply that the Bolwig's Organ mediates the response to light in the Checker Assay and that this response is mediated on behalf of a phototransduction cascade similar to the one operating in the adult. In the ON/OFF assay, *gl* mutants, somda and *pGMR-hid* strains all display a reduction in R.I. and the increase in HS frequency during the light pulse as seen in wild type is abolished (Busto *et al.*, 1999). Thus, the proper development of larval photoreceptors in BO is required for HS and R.I. behaviours in the ON/OFF, and these photoreceptors depend upon an adult-like phototransduction cascade.

An analysis of change of direction behaviour in the ON/OFF assay has revealed that subsets of light response is mediated by a genetically distinct visual system function (Busto *et al.*, 1999). *gl* mutations were found to disrupt only a subset of change of direction responses, whereas mutations in

so abolish magnitude of change of direction at all light transitions. Thus, it was surmised that these change of direction responses are mediated on the part of neurons which do not differentiate under the control of *gl*, but rather under the control of *so* (Busto *et al.*, 1999). In these neurons the response is mediated on behalf of a signaling cascade independent of an adult-like phototransduction cascade because mutations in genes which function in the adult phototransduction cascade do not affect the performance of certain aspects of the change of direction behaviour.

The null allele of *ninaE*, the gene encoding the blue absorbing Rhodopsin, Rh1, was also tested in both assays in order to determine whether Rh1 is required in the larval response to light. Rh1 was found to play no role in mediating light responses (ie. HS and R.I. responses) in photoreceptor cells, which mediate light response on the part of an adult-like phototransduction cascade (Busto *et al.*, 1999). Only subsets of the change of direction responses were abolished: the larva could not distinguish between lights being turned off from the absence of light, and this response is also abolished by *so* but not *gl* mutants. *NinaE* is believed to mediate this distinct visual system in photoreceptor cells whose differentiation is under the control of *so* (Busto et al., 1999).

Other rhodopsin-expressing cells may mediate the response to light. The expression patterns of various rhodopsins in the larval visual system, are currently under investigation and are being conducted using promoter fusions and immunohistochemistry analyses (C. Desplan, personal communication In the past, the expression of Rh1 in subsets of cells in the Bolwig's organ was detected using promoter fusions (Mismer and Rubin, 1987). However, recent re-evaluation of these results has revealed that the detection of Rh1 expression in BO is dependent upon the particular promoter fusion employed (C. Desplan, personal communication). Furthermore, protein expression remains undetected (C. Desplan, personal communication). Rh5 and Rh6 expression have both been detected in promoter fusions, however only Rh5 has been detected through antibody staining. Rh3 and Rh4 are not expressed in contradiction to previous reports (Pollock and Benzer, 1988). No mutations are available in either Rh5 or Rh6, therefore cells were ablated with cell death genes, *rpr* and *hid* to uncover their possible individual roles in light perception.

Ablation Experiments Reveal that Rh5 Mediates Light Responses and that the Integrity of the Remaining Photoreceptor Cells Remain Uncompromised

The ablation of Rh5 expressing photoreceptor cells by the expression of cell death genes *hid* and *rpr* abolishes R.I. and increased frequency of head swinging in the light. This suggests that Rh5 expressing cells mediate the response to light carried out via an adult-like phototransduction cascade. These cells have been previously characterized as photoreceptor cells in BO, which differentiate under the control of the *gl* transcription factor (Busto *et al.*, 1999). The following two issues, must be addressed in order to maintain the validity of these conclusions. These are first, the possible secondary effects of the ablations on the integrity of the remaining photoreceptor cells and second, the extent of cell ablation achieved through ectopic expression of these two cell death genes.

The ablation of Rh5 expressing cells with both *rpr* and *hid* does not affect the integrity of remaining photoreceptor cells. The effects of the ablations on the integrity of the remaining photoreceptor cells was assessed through immunohistochemical analysis. Monoclonal antibody staining with m24B10 of Rh5-*gal4x*UAS-*hid* and Rh5-*gal4x*UAS-*rpr* third instar larval brains showed that both larval optic nerve projection and adult projections were no different from wild type. The extent of ablation on the part of the *hid* gene

appears to be complete. The degree of ablation was assessed through *lacZ* staining of the strain, *Rh5-gal4xUAS-hid*, which had been crossed into a *UAS-tau-lacZ* background. No blue staining was observed indicating that all cells were ablated by *hid*. The control *gl-lacZ* showed blue staining of larval optic nerve and adult projections. Both control and *Rh5-gal4xUAS-hidxUAS-tau-lacZ* were then stained with m24B10 in order to verify that the integrity of the remaining cells were not compromised in these specimens. The negative result (ie. lack of blue staining) must be interpreted with caution as the absence of *UAS-tau-lacZ* or *Rh5-gal4* would yield the same outcome. Therefore, the appropriate controls must be performed in order to assert that ablation is in fact complete. The flies in this particular cross scheme (see APPENDIX C.) which carry only *Rh5-gal4* and *UAS-tau-lacZ* should be tested to make sure that the *UAS-tau-lacZ* has been successfully introduced into the background.

The Role of Rh6 in Larval Response to Light Remains Elusive

The ablation of Rh6 expressing cells by cell death gene *rpr* does not affect the increased frequency of head swinging in the light however, a statistical difference is observed between the R.I.'s of the Rh6 ablated strain and wild

type. Theoretically, if a strain does not respond it should, on average move the same distance in the light as in the dark resulting in a response index of around 0. The R.I. of *Rh6-gal4xUAS-rpr* is 0.2, well above a non-responding strain. It appears that the Rh6 ablated strain is responding, albeit at a reduced level.

An assessment of the integrity of the remaining cells with m24B10 in Rh6-*gal4x*UAS-*rpr*, reveals that the larval optic nerve projection is absent. Some of the Rh6-*gal4x*UAS-*hid* (not tested in this assay) third instar larval brains stained with m24B10 displayed extremely faint staining of the larval optic nerve. Since theoretically in these ablated strains, only 3-4 Rh5-expressing photoreceptor cells should remain it is possible that the cells are under the threshold of visualization. This appears to be a likely explanation in light of the R.I. and HS responses obtained in the ON/OFF assay. The reduced R.I. observed in the assay might be due to aberrant projection patterns of the remaining photoreceptor cells, although this requires further investigation and improved resolution. The extent of ablation along with proper controls should also be further analyzed in both Rh6-*gal4x*UAS-*rpr* and Rh6-*gal4x*UAS-*hid*. The extent of ablation analysis is crucial because a response in the behaviour assay alone is uninformative. This could lead to the faulty conclusion that Rh6-expressing photoreceptor cells are not mediating responses to light in Rh6-*gal4x*UAS-*rpr*, when in fact unablated Rh6 cells

remain and compensate or rescue the response. Thus, the degree of ablation should be assessed in the Rh6 ablated strains.

The Roles of Rh5 and Rh6 in Mediating Change of Direction at Light Transitions Remain Elusive

In all three strains, Rh5-*gal4xUAS-rpr*, Rh5-*gal4x*UAS-*hid* and Rh6-*gal4x*UAS-*rpr* the angle of direction change in larval path at light transitions cannot be compared because the parental control strains, Rh5-*gal4*, Rh6-*gal4*, UAS-*hid* and UAS-*rpr* themselves were unable to distinguish between any of the light transitions. Genetic background of these strains may be hindering an analysis of the change of direction behaviour, thus the strains should be backcrossed in order to circumvent any problems due to the background of the strains.

Recapitulating and Strengthening the Behavioural Analyses: Tetanus toxin, as an Alternative Approach

Confirmation of results from experiments involving the use of *rpr* and *hid* to selectively ablate photoreceptor cells should be sought. Two possible ways of achieving this goal would be by testing other Rh5-*gal4* and Rh6-*gal4*

insertion lines or by ablating the specific Rh5 and Rh6 expressing neurons via alternative mechanisms. On some level, confirmation of the present results has already been actively sought and found. In the case of Rh5, results were reinforced through the testing of two ablated strains using two different cell death genes. Both ablated strains yielded similar results.

Ablations performed with newly constructed Rh1-*gal4* lines (courtesy of J. O'Tousa) should strengthen the present data. Interesting to see whether the results would reinforce ON/OFF assay tests with *ninaE* null mutant strain. Furthermore, it might be interesting to test the ablated strains in the Checker Assay as well to gather more evidence and support for the mediation of an adult-like phototransduction cascade on the part of Rh5-expressing photoreceptor cells.

A completely independent but parallel approach in the ON/OFF assay would involve employing the *GAL4* system to selectively express and impair subsets of neurons. Toxigenic products such as *diphtheria toxin A* and *ricinA*, as temperature sensitive alleles and *ricinA* expressed under a *GAL4*-responsive promoter, UAS, have been employed in *Drosophila* (Kunes and Steller, 1991; Moffat et al., 1992; *Hid*algo et al., 1995; 1997). Selected neurons in the embryonic nervous system as well as neuromuscular junctions have also been successfully ablated using tetanus toxin (Sweeney et al., 1995). The light

polypeptide chain (TetxnLc) cleaves n-synaptobrevin (n-syb), a v-SNARE required for synaptic vesicle exocytosis (Laage *et al.*, 2000). Using the *GAL4* system, the toxin can be targeted to specific neurons, resulting in the elimination of synaptic transmission (Sweeney et al., 1995).

Spectral Sensitivities, a Means Towards Determining the Extent to which Photoreceptor Input is Utilized in Mediating Response to Light as Revealed through our Assays

Mutational analyses and targeted cell ablations continue to be powerful tools towards unraveling the network of molecules and cell types involved in larval response to light. It appears that Rh5-expressing photoreceptor cells are required for mediating both modulation of locomotion (R.I.) and head-swinging behaviours in our ON/OFF Assay. Rh5 and Rh6 have been recently spectrally and photochemically characterized in adult *Drosophila* (Salcedo *et al.*, 1999). Their absorption maximas are at 438 nm and 510nm in the blue and green-wavelength ranges respectively, of the visible spectrum. In the adult, the complete set of rhodopsins Rh1-Rh6 span a broad range of wavelengths from 300-600 nm.

A variety of other organisms have been the focus of wavelength-specific behavioural analyses. Among these are the Australian sheep blowflies,

Lucilia curpina (Fukushi *et al.*, 1985), the swallowtail butterfly, *Papilio xuthus* (Kinoshita *et al.*, 1999), honeybees, *Apis mellifera*, aquatic organisms such as *Daphnia magna* (Storz *et al.*, 1998) and even the marsupial, tammar wallaby or *Macropus eugenii* (Hemmi *et al.*, 1999). Flower visitors, such as the honeybee, depend upon colour vision and display both learned and innate colour preferences. Survival calls for the ability to discriminate between colours as food signals (Lunau *et al.*, 1995) or in avoiding unsafe environments. Colour vision is defined as the ability to identify stimuli solely on the basis of chromatic content and completely independent of intensity. While multiple types of spectral receptors in the eye are most certainly a prerequisite for colour vision, their mere presence is insufficient to conclude that an organism possesses colour vision. The colour vision through behavioural tests and learning paradigms in particular, have helped display the ability to discriminate between various colours (Kinoshita *et al.*, 1999; Fukushi *et al.*, 1985). Different spectral ranges of light may elicit specific behavioural responses, known as wavelength-specific behaviours, however this does not imply that colour vision is necessary for the observed response.

In adult *Drosophila melanogaster*, spectral sensitivities have been successfully employed to determine the extent to which receptor input is utilized in mediating phototactic and optomotor tasks (Hu and Stark, 1977).

In *Drosophila* larva, functions of Rh1 and Rh6 remain elusive, however the spectral specificity of the rhodopsins offers the opportunity to stimulate receptor types individually and observe the direct effect of receptor input on behaviour. Our assays could easily be adapted to undertake a spectral sensitivity analysis of this sort. A Xenon or Mercury-Xenon arc light sources, which possesses a relatively stable output of light ranging from the ultraviolet to the visible region of the spectrum, would illuminate the test plate. Such a light source, along with bandpass filters of narrow bandwidths (approx. 10 nm) along with neutral density filters to adjust light intensity, would permit the larvae to be assayed in the presence of specific wavelengths of light. The breadth of the spectral curve found in *Drosophila*, along with the co-expression of Rh1 and Rh6 poses challenges to a spectral analysis of behaviour. In the larva Rh5, Rh6 and Rh1 all have spectral maxima which are close enough together (438 nm, 475nm and 510nm, respectively) such that individual spectral curves broadly overlap with each other (Salcedo *et al.*, 1999). Even at the maximal peak, it would appear from the breadth of the curve that other rhodopsins may be stimulated as well. Given this observation, in order to substantiate the rolls of Rh5, Rh6 and Rh1 in mediating response to light creating a double mutant (either through ablation or use of null mutants) and stimulating the remaining receptor-type and then to assay subsequent behavioural response. In such a manner the problem of the breadth of the spectral curves may be circumvented.

A genetic strategy involving mutational analyses allowed us to identify two independent visual systems: adult-like phototransduction cascade and another simpler system (Busto *et al.*, 1999). Mutations and targeted ablations have assisted in beginning to define the role of photoreceptor cell-types. The adult-like phototransduction cascade is mediated by Rh5 and operates in photoreceptor cells, which differentiate under the control of the *gl* transcription factor and *so*. The genetic strategies employed thus far, along with wavelength-specific behavioural analyses and large-scale screens for novel larval visual mutants, will assist in continuing to systematically unravel the remaining mysteries of the larval visual system.

Chapter V(APPENDICES): APPENDIX A

The *neither inactivation nor afterpotential C (ninaC)* gene has two photoreceptor specific isoforms, a 132 kD (p132) protein expressed in the cytoplasm and a 174 kD protein (p174) expressed in the rhabdomere (Montell and Rubin, 1988). The proteins consist of a protein kinase and myosin head domain (Montell and Rubin, 1988). These proteins bind calmodulin in the retina and localize it to the rhabdomere and cytoplasm (Porter et al., 1993). Null alleles of *ninaC* have been shown to affect adult phototransduction (Porter et al., 1992; Porter and Montell, 1993).

Previously, two *ninaC* mutants $ninaC^{2}$ and $ninaC^{5}$ were tested in the ON/OFF Assay in order to ascertain whether the gene plays a role in the larval response to light. A wildtype response was reported in $ninaC^{2}$ mutant larvae, however response was abolished in $ninaC^{5}$ as measured by R.I.. The *ninaC* strain has reduced levels of isoform p174 and $ninaC^{5}$ has reduced levels of p174 as well as p132. Thus, it was concluded that the cytoplasmic isoform p132, but not p174 is necessary for light response as measured by the R.I (Busto *et al.*, 1999).

To further the investigate the role of *ninaC* in the larval visual system of Drosophila, several transgenic strains were tested in the ON/OFF

Assay. Among these strains were deletions in single calmodulin binding domains and deletions of single isoforms. Surprisingly, all of these strains displayed a robust response in the assay. The *ninaC*^{2} and *ninaC*^{5} mutants were re-tested along side the transgenic strains and the outcome of these experiments was the same as reported before (Busto *et al.*, 1999). The transgenic flies were all in a null mutant *ninaC*^{P235} background, which is the same allele as the *ninaC*^{5} strain. Surprisingly, the R.I. calculated for the null mutant was no different from wildtype. These, results contradict what was found for *ninaC*^{5}, yet both strains are supposed to be identical. From the tests on the null mutant strain it would appear that *ninaC* is not necessary for larval response to light as measured by R.I. in the ON/OFF Assay.

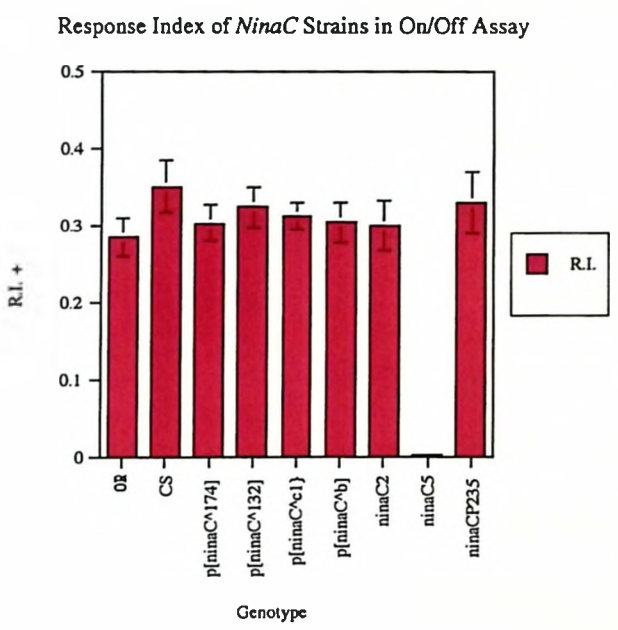

Figure V-1. Response Indices (R.I. ± SEM) for *ninaC* mutant and wild type strains Previous experiments (Busto *et al.*, 1999) as well as repeated experiments shown above with $ninaC^{5}$ (the equivalent of $ninaC^{P235}$ null mutant) indicated that larval response to light as measured by R.I. in the ON/OFF assay is abolished. The $ninaC^{5}$ response was shown to be statistically different from (OR and CantonS) strains. Thus, various trangenes, which are in a null mutant background were further tested. These transgenes included p[$ninaC^{\Delta 174}$] and p[$ninaC^{\Delta 132}$], which possess deleted p174 and p132 isoforms respectively . Strains with a deletion in one of the calmodulin binding domains of *ninaC*, p[$ninaC^{\Delta c1}$] and in both domains, p[$ninaC^{\Delta 8}$] were also tested. All these strains showed a robust response in the ON/OFF assay and their R.I.'s were found not to be significantly different from wild type. Surprisingly, the $ninaC^{P235}$ null mutant shows a robust response as well, which contradicts experimental data from the $ninaC^{5}$ strain.

Appendix B.

The ablation of Rh5 and Rh6-expressing photoreceptor cells via an alternative mechanism proved unsuccessful. The temperature-dependent cell ablations were carried out through the selective expression of a temperature-sensitive *shibire^{ts}* allele. In *shibire* mutants there is a conditional failure of synaptic transmission and Ca^{2+} influx is blocked at nerve terminals (ie. At non-permissive temperatures). The experiments were designed to use the GAL4 system to target the expression of *shibire^{ts}* to these specific photoreceptor cells. Initial control experiments involved growing pGMR-*gal4*xUAS-*shibire^{ts}* at permissive temperature (19°C) and then shifting the larvae to the non-permissive temperature (32°C) for various lengths of time.

Incubation times were varied from 30 minutes to 180 minutes, before testing. Individual larvae were sequentially placed into the water bath (32°C) every 4-5 minutes, such that each larva could be tested in the assay at precisely 30 min, 2hrs or 3 hrs. The R.I in the ON/OFF at the non-permissive temperature was still robust (FigV-2). These control experiments were undertaken to ascertain the minimum incubation time at the higher temperature needed to inactivate targeted neurons through the expression of *shibire^{ts}* allele. This minimum time could then be applied when testing Rh5 and Rh6 ablated

strains. However, control experiments were not successful in abolishing response to light as measured by R.I. (Fig. V-2).

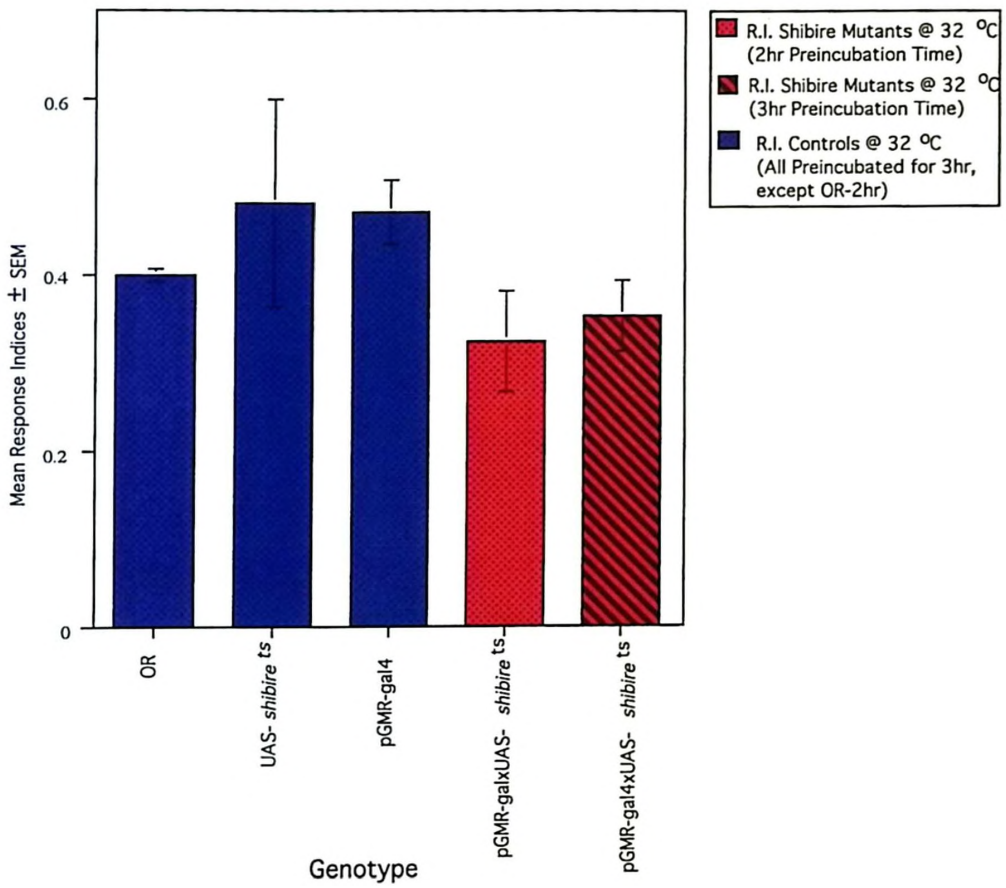

Figure V-2 .
Mean Response Indices (R.I.'s)for Controls and Temperature-sensitive *shibire* mutants
The control strains, wild type and parental strains grown at the permissive temperature and then pre-incubated at non-permissive temperature of 32 degrees Celcius before assay, show a robust response in the ON/OFF assay. The larval progeny, in which the expression of the temperature-sensitive allele is targeted to *gl-* expressing cells, continue to show a robust response at both 2 hr and 3 hr pre-incubation times at the non-permissive temperature.

Appendix C.

$$\frac{+}{+};\frac{Sp}{CyO};\frac{Rh5\text{-}gal4}{Rh5\text{-}gal4} \times \frac{+}{y};\frac{S}{CyO};\frac{CxD}{TM9.Sb}$$

$$\frac{+}{y};\frac{S}{CyO};\frac{Rh5\text{-}gal4}{TM9.Sb}$$

$$\frac{+}{+};\frac{Sp}{CyO};\frac{Rh5\text{-}gal4}{Rh5\text{-}gal4} \times \frac{+}{y};\frac{S}{CyO};\frac{Rh5\text{-}gal4}{TM9.Sb}$$

$$\frac{+}{y};\frac{S}{CyO};\frac{Rh5\text{-}gal4}{Rh5\text{-}gal4}$$

$$\frac{+}{+};\frac{UASg\text{-}hid}{UASg\text{-}hid};\frac{+}{+} \times \frac{+}{y};\frac{S}{CyO};\frac{CxD}{TM9.Sb}$$

$$\frac{+}{+};\frac{UASg\text{-}hid}{CyO};\frac{+}{TM9.Sb}$$

$$\frac{+}{+};\frac{UASg\text{-}hid}{CyO};\frac{+}{TM9.Sb} \times \frac{+}{y};\frac{S}{CyO};\frac{Rh5\text{-}gal4}{Rh5\text{-}gal4}$$

$$\frac{+}{+};\frac{UASg\text{-}hid}{CyO};\frac{Rh5\text{-}gal4}{TM9.Sb}$$

$$\frac{+}{+};\frac{\text{UASg-hid}}{\text{CyO}};\frac{\text{Rh5-gal4}}{\text{TM9.Sb}} \times \frac{+}{+};\frac{\text{UASg-hid}}{\text{CyO}};\frac{\text{Rh5-gal4}}{\text{TM9.Sb}}$$

$$\frac{+}{+};\frac{\text{UASg-hid}}{\text{UASg-hid}};\frac{\text{Rh5-gal4}}{\text{Rh5-gal4}}$$

$$\frac{+}{+};\frac{\text{UASg-hid}}{\text{UASg-hid}};\frac{\text{Rh5-gal4}}{\text{Rh5-gal4}} \times \frac{+}{+};\frac{+}{+};\frac{\text{UAS-lacZ}}{\text{UAS-lacZ}}$$

$$\frac{+}{+};\frac{\text{UASg-hid}}{+};\frac{\text{Rh5-gal4}}{\text{UAS-lacZ}}$$

Figure V-3:
Cross Scheme: Rh5-ablation in UAS-*tau-lacZ* backround
The above cross scheme was followed in order to obtain larvae in which Rh5-*gal4* and UASg-*hid* were in a UAS-tau-lacZ background. The cross was performed in order to determine the extent of ablation of Rh5-expressing cells.

References

Aceves-Pina, E. O., Quinn W., Smith K., Steinberger E., and Rodriguez-Rigau, L. J. (1979). Learning in Normal and Mutant *Drosophila* Larvae. Science 206, 93-96.

Anholt, R. R. H, Lyman, R. F., and Mackay, T. F. C. (1996). Effects of Single P-Element Insertions on Olfactory Behavior in *Drosophila melanogaster.* Genetics 143, 293-301.

Ashburner, M. (1989). Developmental biology. In: *Drosophila*: A laboratory handbook. Cold Spring Harvor, NY: Cold Spring Harbor Laboratory.
Dunlap, J.C. (1999). Molecular Bases for Circadian Clocks. Cell 96, 271-290.

Avery, L. and Horovitz, H. R. (1987). A cell that dies during wild-type *C. elegans* development can function as a neuron in a *ced-3* mutant. Cell. 51, 1071-1078.

Bloomquist, B. T., Shortridge, R. D., Schneuwly, S., Perdew, M., Montell, C., steller, H., Rubin, G., Pak, W. L. (1988). Isolation of a Putative Phospholipase C Gene of *Drosophila, norpA,* and Its Role in Phototransduction. Cell. 54, 723-733.

Bolwig, N., (1946). Sense and sense organs by the anterior end of the house fly larvae. Vidensk Medd Dan Naturhist Foren. 109, 81-217.

Brand, A. H., and Manoukian, A. S., and Perrimon, N. (1994). Ectopic Expression in *Drosophila.* Methods in Cell Biology. 44, 635-654.

Brand, A. H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 118, 401-415.

Brand, A. H., and Dormand, E. (1995). The GAL4 system as a tool for unravelling the mysteries of the *Drosophila* nervous system. Curr. Opin. Neurobio. 5, 572-578.

Browder, L. W. (1980). Developmenal Biology. Philadelphia: Saunders College.

Burnet, B. and Kyriacou, C. P. (1979). Genetic Analysis of Phototaxis Near the Upper Limit of the Visual Spectrum of *Drosophila melanogaster*. Behav. Genetics. 9(2), 123-128.

Busto, M., Iyengar, B. G., and Campos, A. R. (1999). Genetic Dissection of Behavior: Modulation of Locomotion by Light in the *Drosophila melanogaster* Larva Requires Genetically Distinct Visual System Functions. J. Neurosc. 19(9), 3337-3344.

Callahan, C. A., and Thomas, J. B. (1994). Tau-β-galactosidase, an axon-targeted fusion protein. Proc. Natl. Acad. Sci. USA. 91, 5972-5976.
Lilly, M. and Carlson, J. (1989). *smellblind*: A Gene Required for *Drosophila* Olfaction. Genetics 124, 293-302.

Campos, A. R., Lee, K. J., and Steller, H. (1995). Establishment of neuronal connectivity during development of the *Drosophila* larval visual system. J. Neurobiol. 28, 313-329.

Chalfie, M. and Sulston, J. (1981). Developmental genetics of the mechanosensory neurons of *Caenorhabditis elegans*. Dev. Biol. 82, 358-370.

Cheyette, B. N. R., Green, P. J., Martin, K., Garren, H., Hartenstein, V., and Zipursky, S. L. (1994). The Drosoophila *sine oculis* Locus Encodes a Homeodomain-Containing Protein Required for the Development of the Entire Visual System. Neuron. 12, 977-996.

Chou, W. H., Hall, K. J., Wilson, D. B., Wideman, C. L., Townson, S.M., Chadwell, L. V., and Britt, S. G. (1996). Identification of a novel *Drosophila* opsin reveals specific paterning of the R7 and R8 phototreceptor cells. Neuron. 17, 1101-1115.

Chou. W., Huber, A., Bentrop, J., Schutz, S., Schwab, K., Chadwill, L.V., Paulsen, R., Britt, S. G. (1999). Patterning of the R7 and R8 photoreceptor cells of *Drosophila*: evidence for induced and default cell-fate specification. Development. 126, 607-616.

Chyb, S., Raghu, P., and Hardie, R. C. (1999). Polyunsaturated fatty acids activate the *Drosophila* light-sensitive channels *TRP* and *TRP*L. Nature. 397, 255-258.

Dunlap, J. C. (1999). Molecular bases for Circadian *Clock*s. Cell. 96, 271-290.

Drzislav, M., and Rubin, G. M. (1989). Definition of cis-Acting Elements Regulating Expression of the *Drosophila melanogaster ninaE* Opsin Gene by Oligonucleotide-Directed Mutagenesis. Genetics. 121, 77-87.

Dyer, A. G. (1999). Broad spectral sensitivities in the honeybee's photoreceptors limit colour constancy. J. Comp. Physiol. A. 185, 445-453.

Eberl, D. F., Duyk, G. M., and Perrimon, N. (1997). A genetic screen for mutations that disrupt an auditory response in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 94, 14837-14842.

Feiler, R., Bjornson, R., Kirschfeld, K., Mismer, D., Rubin, G. M., Smith, D. P., Socolich, M., and Zuker, C. S. (1992). Ectopic Expression of Ultraviolet-Rhodopsins in the Blue Photoreceptor Cells of *Drosophila*: Visual Physiology and Photochemistry of Transgenic Animals. J. Neurosc. 12(10, 3862-3868.

Fukushi, T. (1985). Visual learning in walking blowflies, Lucilia cuprina. J. Comp. Physiol. A. 157, 771-778.

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

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

Godoy-Herrera, R., Santander, R., and Figueroa, J. (1994). A developmental and biometrical analysis of larval photoresponse of *Drosophila*. Anim. Behav. 48, 251-262.

Hall, J. C. (1977). Portions of the central nervous system controlling reproductive behavior in *Drosophila melanogaster*. Behav. Genetics, 7(4), 291-312.

Hardie, R. C., and Minke, B. (1992). The *trp* Gene Is Essential for a Light-Activated Ca^{2+} Channel in *Drosophila* Photoreceptors. Neuron. 8, 643-651.

Hassan, J., Busto, M., Iyengar, B., and Campos, A. R. (2000). Behavioral Characterization and Genetic Analysis of the *Drosophila melanogaster* Larval Response to Light as Revealed by a Novel Individual Assay. Behav. Genetics. 30(1), 59-69.

Heisenberg, M., and Buchner, E. (1977). The Role of Retinula Cell Types in Visual Behavior of *Drosophila melanogaster*. J. Comp. Physiol. 117, 127-162.

Hemmi, J. M. (1999). Dichromatisc colour vision in an Australian marsupial, the tammar wallaby. J. Comp. Physiol. A. 185: 509-515.

*Hid*algo, A., Urban, J., and Brand, A. H. (1995). Targeted ablation of glia disrupts axon tract formation in the *Drosophila* CNS. Development 121, 3703-3712.

*Hid*algo, A., and Brand, A. H. (1997). Targeted neuronal ablation: the role of pioneer neurons in guidance and fasciculation in the CNS of *Drosophila*. Development. 124, 3253-3262.

Hofstee, C. A., Henderson, S., Hardie, R. C., and Stavenga, D. G. (1996). Differential effects of *ninaC* proteins (p132 and p174) on light-activated currents and pupil mechanism in *Drosophila* photoreceptors. Vis. Neurosc. 13, 897-906.

Hotta, Y., and Benzer, S. (1970). Genetic Dissection of the *Drosophila* Nervous System by Means of Mosaics. Proc. Natl. Acad. Sci. 67(3), 1156-1163.

Hu, K. G. and Stark, W. S. (1977). Specific Receptor Input into Spectral Preference in *Drosophila*. J. Comp. Physiol. 121, 241-252.

Hu, K. G., and Stark, W. S. (1980). The Roles of *Drosophila* Ocelli and Compound Eyes in Phototaxis. J. Comp. Physiol. 135, 85-95.

Huber, A., Schulz, S., Bentrop, J., Groell, C., Wolfrum, U., Paulsen, R. (1997). Molecular cloning of *Drosophila* Rh6 rhodopsin: the visual pigment of a subset of R8 photoreceptor cells. FEBS Lett. 406(1-2), 6-10.

Ito, K., Sass, H., Urban, J., Hofbauer, A., and Schneuwly, S. (1997). GAL4-responsive *UAS-tau* as a tool for studying the anatomy and development of the *Drosophila* central nervous system. Cell Tissue Res. 290, 1-10.

Iyengar, B., Roote, J., and Campos, A. R. (1999). The tamas Gene, Identified as a Mutation That Disrupts Larval Behavior in *Drosophila melanogaster*, Codes for the Mitochondrial DNA Polymerase Catalytic Subunit (DNApol-γ125). Genetics. 153, 1809-1824.

Kinoshita, M., Shimada, N., and Arikawa, K. (1999). Colour Vision of the Foraging Swallowtail Butterfly Papilio Xuthus. J. Exp. Bio. 202, 94-102.

Kunes, S., and Steller, H. (1991). Ablation of *Drosophila* photoreceptor cells by conditional expression of a toxin gene. Genes&Dev. 5, 970-983.

Laage, R., Rohde, J., Brosig, B. and Langosch, D. (2000). A Conserved Membrane-Spanning Amino Acid Motif Drives Homomeric and Supports Heterotrimeric Assembly of Presynaptic SNARE Proteins. J Biol. Chem. 3, 1-20.

Lee, K. J., Freeman, M., and Steller, H. (1991). Expression of the disconnected gene during development of *Drosophila melanogaster*. EMBO. 10(4), 817-826.

Lunau, K., and Maier, E. J. (1995). Innate colour preferences of flower visitors. J. Comp. Physiol. A. 177, 1-19.

McNabb, S. L, Baker, J. D., Agapite, J., Steller, H., Riddford, L. M and Truman, J. W. (1997). Disruption of a Behavioral Sequence by Targeted Death of Peptidergic Neurons in *Drosophila*. Neuron. 19, 813-823.

Menzel, M. and Muller, U. (1996). Learning and Memory in Honeybees: From Behavior to Neural Substrates. Annu. Rev. Neurosci. 19, 379-404.

Meyerowitz, E. M., and Kankel, D. R. (1978). A Genetic Analysis of Visual System Development in *Drosophila melanogaster*. Dev. Biol. 62, 112-142.

Miller, G. V., Hansen, K. N., and Stark, W. S. (1981). Phototaxis in *Drosophila*: R1-6 Input and Interaction Among Ocellar and Compound Eye Receptors. J. Insect Physiol. 27(11), 813-819.

Mismer D. and Rubin, R. M. (1989) Definition of cis-Acting Elements Regulating Expression of the *Drosophila melanogaster ninaE* Opsin Gene by Oligonucleotide-Directed Mutagenesis. Genetics. 121, 77-87.

Moffat, K. G., Gould, J. H., Smith, H. K., and O'Kane, C. J. (1992). Inducible cell ablation in *Drosophila* by cold-sensitive *ricin A* chain. Development. 114, 681-687.

Montell, C. (1999). Visual Transduction in *Drosophila*. Annu. Rev. Cell Dev. Biol. 15, 231-268.

Montell, C., and Rubin, G. M. (1989). Molecular Characterization of the *Drosophila trp* Locus: A Putative Integral Membrane Protein Required for Phototransduction. Neuron. 2, 1313-1323.

Montell, C., Jones, K., Zuker, C., and Rubin, G. (1987). A Second Opsin Gene Expressed in the Ultraviolet-Sensitive R7 Photoreceptor Cells of *Drosophila melanogaster*. J. Neurosc. 7(5), 1558-1566.

Morrison, G. E., Wen, J. Y. M., Runciman, S., and van der Kooy, D. (1999). Olfactory Associative Learning in *Caenorhabditis elegans* is Impaired in *lrn-1* and *lrn-2* Mutants. Behav. Neurosc. 113, 358-367.

O'Tousa, J., Baehr, W., Martin, R. L., Hirsh, J., Pak, W. L., Applebury, M. L. (1985). The *Drosophila ninaE* Gene Encodes an Opsin. Cell. 40, 839-850.

Pak, W., Grossfield, J., White, N. V. (1969). Non phototactic mutants in a study of vision of *Drosophila*. Nature. 222, 351-354.

Pak, W. L. (1995). *Drosophila* in Vision Research. Inves. Ophtal.&Vis. Sci. 36(12), 2340-2357.

Papatsenko, D., Sheng, G., and Desplan. (1997). A new rhodopsin in R8 photoreceptors of *Drosophila*: evidence for coordinate expression with Rh3 in R7 cells. Development. 124, 1665-1673.

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F., and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. J. Comp. Physiol. 170, 23-40.

Phelps, C. B., and Brand, A. H. (1998). Ectopic Gene Expression in *Drosophila* Using GAL4 System. Methods 14, 367-379.

Pollock, J. A., and Benzer, S. (1988). Transcript localization of four opsin genes in the tree visual organs of *Drosophila*; RH2 is ocellus specific. Nature. 333, 779-782.

Porter, J. A., and Montell, C. (1993). Distinct Roles of the *Drosophila* ninaC Kinase and Myosin Domains Revealed by Systematic Mutagenesis. J. Cell Biol. 122(3), 601-612.

Renn, S. C., Park, J. H., Rosbash, M., Hall, J. C., and Taghert, P. H. (1999). A pdf Neuropeptide Gene Mutation and Ablation of PDF Neurons Each Cause Severe Abnormalities of Behavioral Circadian Rhythms in *Drosophila*. Cell. 99, 791-802.

Sawin-McCormack, E., Sokolowski, M. B., and Campos, A. R. (1995). Characterization and Genetic Analysis of *Drosophila melanogaster* Photobehavior during Larval Development. J. Neurogenet. 10, 119-135.

Smith, D. P., Stammes, M. A., and Zuker, C. S. (1991). Signal Transduction in the Visual System of *Drosophila*. Annu. Rev. Cell Biol. 7, 161-190.

Salcedo, E., Huber, A., Henrich, S., Chadwell, L. V., Chou, W., Paulsen, R., and Britt, S. G. (1999). Blue- and Green-Absorbing Visual Pigments of *Drosophila*: Ectopic Expression and Physiological Characterization of the R8 Photoreceptor Cell-Specific Rh5 and Rh6 Rhodopsins. J. Neurosci. 19(24), 10716-10726.

Schmucker, D., Jaeckle, H., and Gaul, U. (1997). Genetic analysis of the larval optic nerve projection in *Drosophila*. Development. 124, 937-948.

Schmucker, D., Taubert, H., and Jaeckle, H. (1992). Formation of the *Drosophila* Larval Photoreceptor Organ and Its Neuronal Differentiation Require Continuous *Kruppel* Gene Activity. Neuron. 9, 1025-1039.

Scott, K., Sun, Y., Beckingham, K., and Zuker, C. (1997). Calmodulin Regulation of *Drosophila* light-Activated Channels and Receptor Function Mediates Termination of the Light Respsonse In Vivo. Cell. 91, 375-383.

Serikaku, M. A., and O'Tousa, J. E. (1994). sine oculis Is a Homeobox Gene Required for *Drosophila* Visual System Development. Genetics. 138, 1137-1150.

Smith, L. A., Peixoto, A. A., Kramer, E. M., Villella, A., and Hall, J. C. (1988). Courtship and Visual Defects of cacophony Mutants Reveal Functional Complexity of a Calcium-Channel $\alpha 1$ Subunit in *Drosophila*. Genetics 149, 1407-1426.

Sokolowski, M. B., Kent, C., and Wong, J. (1984). *Drosophila* Larval Foraging Behaviour: Developmental Stages. Anim. Behav. 32, 645-651.

Stark, W. (1975). Spectral Selectivity of Visual Response Alterations Mediated by Interconversions of Native and Intermediate Photopigments in *Drosophila*. J. Comp. Physiol. 96, 343-356.

Steller, H., Fischbach, K., and Rubin, G. M. (1987). disconnected: A Locus Required for Neuronal Pathway Formation in the Visual System of *Drosophila*. Cell. 50, 1139-1153.

Storz, U. C., and Paul, R. J. (1998). Phototaxis in water fleas (Daphnia magna) is differently influenced by visible and UV light. J. Comp. Physiol A. 183, 709-717.

Sweeney, S. T., Broadie, K., Keane, J., Niemann, H., and O'Kane, C. J. (1995). Targeted Expression of Tetanus Toxin Light Chain in *Drosophila* Specifically Eliminates Synaptic Transmission and Causes Behavioral Defects. Neuron 14, 341-351.

Townson, S. M., Chang, B. S. W., Salcedo, E., Chadwell, L. V., Pierce, N. E., and Britt, S. G. (1998). Honeybee Blue- and Ultraviolet-Sensitive Opsins: Cloning, Heterologous Expression in *Drosophila* and Physiological Characterization. J. Neurosci. 18(7), 2412-2422.

Tully, T., Preat., T., Boynton, S. C., and Del Vecchio, M. (1994). Genetic Dissection of Consolidated Memory in *Drosophila*. Cell 79, 35-47.

Umbach, J. A., Saitoe, M., Kidokoro, Y. and Gunderson, C. B. (1998). Attenuated Influx of Calcium Ions at Nerve endings of *csp* and *shibire* Mutant *Drosophila*. J. Neurosc. 18(9), 3233-3240.

Van Vactor, D. Jr., Krantz, D. E., Reinke, R., and Zipursky, S. L. (1988). Analysis of Mutants in Chaoptin, a Photoreceptor Cell-Specific Glycoprotein in *Drosophila*, Reveals Its Role in Cellular Morphogenesis.
Cell. 52, 281-290.

Vitaterna, M. h., King, D. P., Chang, A. M., Kornahauser, J. M., Lowrey, P. L. (1994). Mutagenesis and mapping of a mouse gene, *clock*, essential for circadian behavior. Science. 264, 719-725.

Wen, J. Y. M., Kumar, N., Morrison, G., Rambaldini, G., Runciman, S., Rousseau, J. and van der Kooy, D. Mutations That Prevent Associative Learning in C. Elegans. Behav. Neurosc. 111(2), 354-368.

White, K., Tahaoglu, E., and Steller, H. (1996). Cell Killing by the *Drosophila* gene *reaper*. Science. 271, 805-807.

Yang, M. Y., Armstrong, J. D., Vilinsky, I., Strausfeld, N. J., and K. Kaiser (1995). Subdivision of the *Drosophila* Mushroom bodies by Enhancer-Trap Expression Patterns. Neuron 15, 45-54.

Zhou, L., Schnitzler, A., Agapite, J., Schwartz, L. M., Steller, H., and Nambu, J. R. (1997). Cooperative functions of the *reaper* and *head involution defective* genes in the programmed cell death of *Drosophila* central nervous system midline cells. Proc. Natl. Acad. Sci. USA 94, 5131-5136.

Zipursky, S. L., Venkatesh, T. R., Teplow, D. B., and Benzer, S. (1984). Neuronal Development in the *Drosophila* Retina: Monoclonal Antibodies as Molecular Probes. Cell. 36, 15-26.