GENETIC DISSECTION OF THE *D. MELANOGASTER* LARVAL RESPONSE TO LIGHT

GENETIC DISSECTION OF THE *DROSOPHILA MELANOGASTER* LARVAL RESPONSE TO LIGHT MEASURED IN TWO NEW SINGLE LARVA ASSAYS

By

MACARENA M. BUSTO, B.Sc.

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AUTHOR: Macarena M. Busto, B.Sc. (University of Western Ontario)

SUPERVISOR: Dr. Ana R. Campos

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Abstract

In order to initiate a genetic dissection of the *Drosophila melanogaster* larval response to light, two new single larva assays were designed: the Checker and ON/OFF assays. Each assay allows quantification of different aspects of the larval visual response by permitting the study of discrete behaviours in a single larva. Results of this study indicate that larvae respond to light by modulating their locomotion. In the Checker assay this can be seen as an increase in residence time spent in dark checks. In the ON/OFF assay this can be measured as a decrease in distance travelled during the light pulse, due at least in part to an increase in head swinging. Concomitantly, the larva exhibits a sharp change in direction from its original path when the lights are turned on. When the lights are turned off, the change in direction in the larval path, although smaller than at lights on, is still greater than in the absence of light transitions. Many of the components previously described to function in adult phototransduction and visual system specification, also have roles in the larval photoresponse as mutations in the genes that encode these components, are able to abolish light perception as measured in both the Checker and ON/OFF assays. However, these mutations disrupt only subsets of the behaviours associated with the larval perception of light, thus suggesting the existence of light detecting mechanism independent of the main visual pathway described for the adult visual system.

Acknowledgements

I would like thank my supervisor, Dr. Ana Campos, for her support and guidance throughout this undertaking.

Also, to all the members of the Campos lab: thanks for your friendship, for making me laugh, for listening to me rant and rave and finally for making this a time I will look back on fondly.

To my family: thank you for always and unfailingly supporting and encouraging me even when you weren't quite sure what it was I was doing or why I was doing it. Your faith in me has made it possible.

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Chapter I

Introduction

Thirty years have passed since Seymour Benzer isolated, using behavioural paradigms, the first *Drosophila* phototaxis mutants (Benzer, 1967). Since that time, the number of researchers and the volume of literature, on phototaxis in particular and *Drosophila melanogaster* in general, has grown almost exponentially. The reasons why *Drosophila* remains a favoured model organism today are the same as they were for Benzer in the 1960's; a broad information base and a number of molecular and genetic techniques unavailable in other organisms.

The use of *Drosophila* as a model organism for genetic analysis has a number of advantages: a short generation time (10 - 14 days), prodigious fertility and a small chromosome complement (1 sex chromosome and 3 autosomes) (reviewed in Sokolowski, 1992). The *Drosophila* life cycle can be divided into several distinct developmental stages; embryo, larva, pupa and adult. Each of these has been further subdivided, in the case of larvae into instars, by the presence of anatomical or physical landmarks. In brief, *Drosophila* is large enough to have a sophisticated nervous system and a range of behaviours, without being too complex. It is midway between the complexity seen in *Escherichia coli* and humans (Greenspan, 1990).

It is defects in this range of behaviours which was initially exploited by the pioneers in the field of neurogenetics and which continue to yield interesting mutants. The rationale was to isolate single gene mutations affecting specific behaviours in the hope that these mutants might define 'steps' in a 'pathway' (Greenspan, 1990). It is a strategy which has proven successful time and again. In this manner mutations affecting circadian rhythms (Konopka and Benzer, 1971; Dushay *et al*, 1990; Konopka *et al*, 1991; Newby and Jackson, 1993; Matsumoto *et al*, 1994; Sehgal *et al*, 1994; Murata *et al*, 1995), learning (Dudai *et al*, 1976; Phelan *et al*, 1998; Tempel *et al*, 1983; Aceves-Pina and Quinn, 1979; Boynton and Tully, 1992) olfaction (Fuyama, 1976; Rodrigues and Siddiqi, 1978; Aceves-Pina and Quinn, 1979; Siddiqi, 1987; Helfand and Carlson, 1989; Woodard *et al*, 1991; Lilly and Carlson, 1990; Anholt *et al*, 1996), phototransduction and visual system assembly (Benzer, 1967; Pak *et al*, 1969; Hotta and Benzer, 1970; Pak et al, 1970; Harris *et al*, 1976; Kyriacou and Burnet, 1979; Gerresheim, 1988; Gordesky-Gold et al, 1995) were first isolated. Fortunately, the work did not stop here.

As a result of much of the work mentioned above, a number of mutants has been isolated in screens for flies with defects in vision. With the advent of gene cloning and sequencing, it became possible to find the molecular basis for these behavioural deficits. As a result, many genes associated with the behavioural mutations have been shown to function in adult phototransduction (reviewed in Ranganathan *et al*, 1995) and adult visual system assembly (i.e. Banerjee *et al*, 1987; reviewed in Freeman, 1997). However, not much information is available about larval visual system function.

Drosophila has two visual systems: one present in the larva and one present in adult flies. The adult visual system is comprised of the compound eyes, ocelli and optic lobes. The main adult visual organ, the compound eye is made up of 800 repeating units, or ommatidia. Each ommatidium is itself composed of 8 photoreceptor and 12 accessory cells (reviewed in Freeman, 1997). Named for their position in the ommatidium and not the sequence in which they arise, the photoreceptors (R1 - R8) lie at the centre of the ommatidium. From these photoreceptors extends a photosensitive stack of microvilli, called a rhabdomere, into the extracellular space of the central canal. They are arranged such that photoreceptors R1 - R6 occupy outer positions and R7 and R8 occupy central positions.

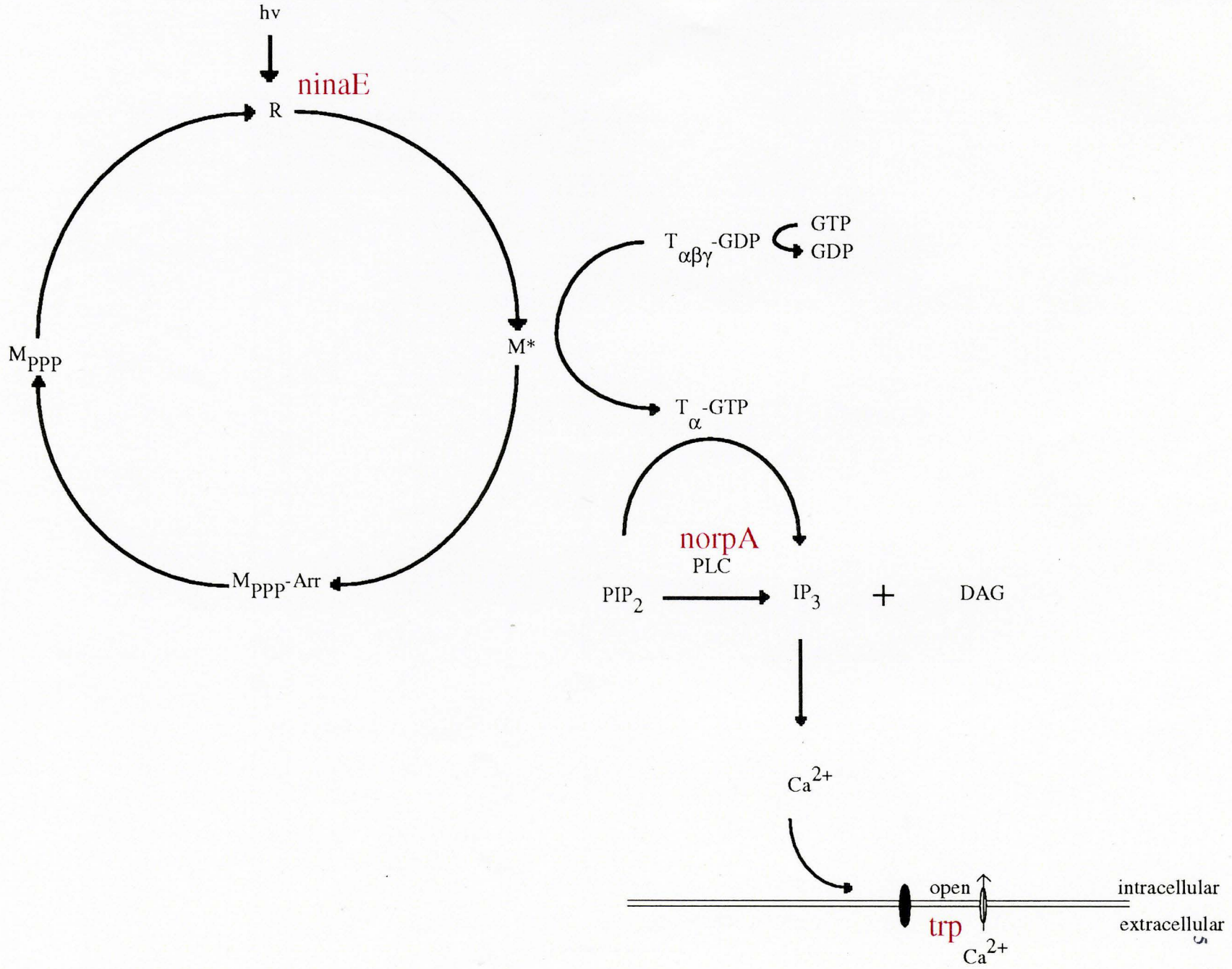
In addition to their position, the photoreceptors can be further subdivided into three distinct groups based on morphological and functional information. Photoreceptors R1 - R6 represent the major class of photoreceptors in the adult eye and are maximally sensitive to blue light. The axons originating from these photoreceptors synapse onto neurons in the lamina. Photoreceptor cell R7, located in the distal portion of the ommatidium is maximally sensitive to ultraviolet light. The R8 photoreceptor cell, located in the proximal portion of the ommatidium, is sensitive to blue-green light. Axons of both R7 and R8 proceed past the lamina and synapse at the medulla (reviewed in Freeman, 1997).

Phototransduction in the adult eye is accomplished by a series of biochemical reactions. During these reactions light energy is converted into an electrophysiological response. A diagrammatic, summarized representation of the phototransduction cascade

in the *Drosophila* compound eye is shown in Figure 1 (reviewed in Ranganathan *et al*, 1995 and Zucker, 1996).

The light receptor molecule rhodopsin (R) is composed of an opsin molecule covalently linked to a chromophore. It is the opsin molecule which confers spectral sensitivity to rhodopsin. Light absorption by the chromophore moiety triggers a conformational change in protein. This activated rhodopsin or metarhodopsin (M) then activates a heterotrimeric GTP-binding protein (G protein). This G protein in turn activates a phospholipase C (PLC) encoded by the *norpA* gene (Bloomquist *et al*, 1988). PLC catalyzes the breakdown of phosphotidyl inositol 4,5-bisphosphate (PIP_2) into the two intracellular messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mediates the release of intracellular calcium through cation channels, which in turn leads to the generation of a depolarizing receptor potential (Berridge and Irvine, 1984). DAG activates a protein kinase C (PKC) which is thought to function by phosphorylating rhodopsin or other members of the phototransduction cascade in order to regulate their activity (Schaeffer *et al*, 1989)

In contrast to the complex organization seen in the adult eye, the larval visual system is fairly simple. Originally described by Bolwig (1946) in fly *Musca domestica*, features of the *Drosophila* system were described by analogy to the larger fly. The larval visual organs, named Bolwig's organs are two bilaterally symmetrical groups of approximately 12 photoreceptor cells, which are located just anterior to the cephalopharyngeal skeleton (Steller *et al*, 1987). These cells arise during embryogenesis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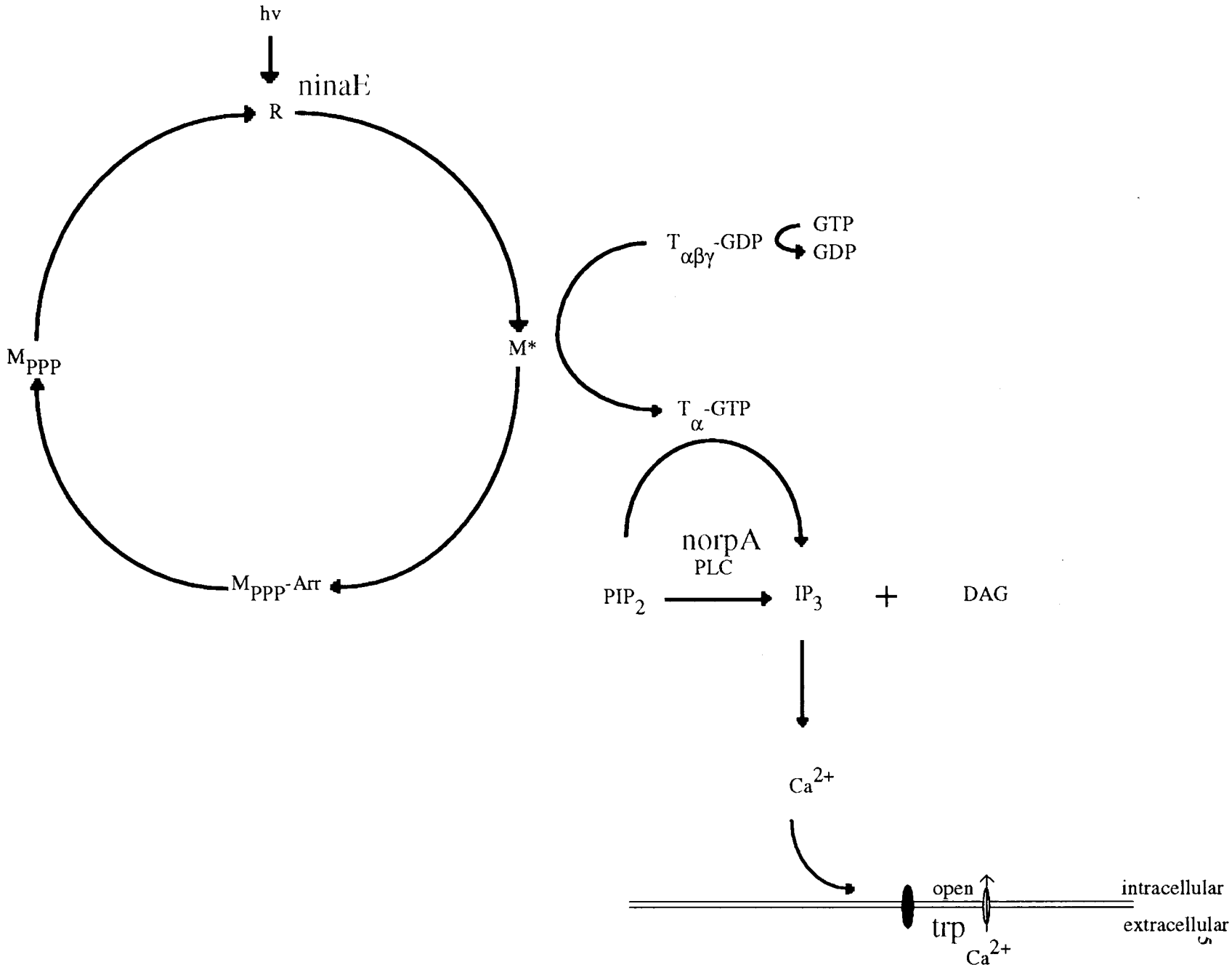
GTP
GDP
T_{α}-GTP
norpA
PLC
PIP_2
IP_3
+
DAG
Ca^{2+}
open
trp
intracellular
extracellular
Ca^{2+}

Figure 1. The invertebrate phototransduction cascade in *Drosophila* adult photoreceptor cells

Schematic representation of a simplified version of the signal transduction cascade in adult photoreceptor cells which results in a response to light. Absorption of light causes a conformational change in rhodopsin (R), the opsin moiety of which is encoded by the *ninaE* gene in R1-R6 photoreceptor cells, and activates it. Active metarhodopsin (M) catalyzes G protein activation such that the G protein exchanges guanosine diphosphate (GDP) for guanosine triphosphate (GTP) and releases its inhibitory $\beta\gamma$ subunits. The active G protein catalyzes activation of phospholipase C (PLC), encoded by the *norpA* gene, which then hydrolyzes phosphotidyl inositol biphosphate (PIP_2) into the intracellular messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mediates the release of intracellular Ca^{2+} through cation channels, one type at least which is encoded by the *trp* gene, such that a depolarizing receptor potential is generated. Adapted from Zucker, 1996.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and establish contacts with the optic lobes (Steller *et al*, 1987; Schmucker *et al*, 1992; Green *et al*, 1993; Campos *et al*, 1995; Schmucker *et al*, 1997).

The cells which form the Bolwigs organ and optic lobe anlagen, the primordia of the adult optic lobes, appear to arise from a common pool of precursor cells (Schmucker *et al*, 1997). These two cell populations are initially very close spatially and become separated during the process of head involution during embryogenesis (Schmucker et al, 1997). During this time the axons which form Bolwigs nerve extend to meet the demand of increased distance between the Bolwigs organs and the targets in the optic lobes and central brain. The *sine oculis* (*so*) gene which is required for visual system specification is expressed in both sets of precursor cells (Cheyette *et al*, 1994; Serikaku and O'Tousa, 1994). Expression of *so* precedes expression of several other genes involved in visual system function and assembly (Serikaku and O'Tousa, 1994). One of these, *glass* (*gl*), is required for the proper differentiation of the Bolwigs organ cells into photoreceptors (Moses *et al*, 1989; Schmucker *et al*, 1992; Campos *et al*, 1995).

Axons originating from these photoreceptor cells fasciculate together to form the larval Bolwig's nerve, which connects with target neurons located in the optic lobes. Reminiscent of the situation in the compound eye where axons originating from R7, R8 and R1 - R6 synapse at different levels, so do larval photoreceptor axons. Some of the Bolwig's nerve fibres synapse in the central brain while others have more peripheral sites of termination within the optic lobes (Tix *et al*, 1989). The functional importance of the differential targeting by the axons is unknown. The embryonic development of the larval visual system has been previously described (Green *et al*, 1993; Campos *et al*, 1995).

Spectral sensitivity studies of larval photoreceptors have not been done. However, Pollock and Benzer (1988), demonstrated that Rh3 and Rh4, both of which are maximally sensitive to UV, are expressed in the larva. Although larval expression patterns of Rh5 and Rh6, maximally sensitive to blue-green light, have not yet been studied, they are also presumably expressed in larval photoreceptors. The possibility also exists that larval photoreceptors express not yet identified rhodopsins specific to that structure, much like Rh2 is expressed only in the ocelli of adult flies (Pollock and Benzer, 1988).

The lack of complexity in the larval light sensing organs is understandable when one considers the larval life cycle. The larval stage is divided into three larval instars separated by two molts during which time the larva maintains a similarly organized sensory system (Demerec and Kaufman, 1940; Grossfield, 1978). During its life span, in the presence of food the *Drosophila* larva, spends much of its time feeding (Bakker, 1961; Green *et al*, 1983; Godoy-Herrera *et al*, 1994). During this time the larva is repelled by light (Hotta and Keng, 1984; Lilly and Carlson, 1990; Gordesky-Gold *et al*, 1995; Sawin-McCormack *et al*, 1995). It has been reported that late in third instar however, this repulsion to light is reversed such that the larva is now attracted to light (Godoy-Herrera *et al*, 1992). This coincides with the onset of wandering during which time the larva leaves the food substrate in search of a suitable pupation site (Sokolowski *et al*, 1984; Godoy-Herrera *et al*, 1989). Other reports however have not confirmed this change from light repulsion to light attraction (Sawin-McCormack *et al*. 1995).

How the larva responds to light is not well understood. Although genes expressed in both larval and adult visual systems have been identified including a subset of

rhodopsins and *gl*, it is unknown whether these genes have identical functions in each system (Pollock and Benzer, 1988; Moses *et al*, 1989). In order to initiate a genetic dissection of the larval visual response, two new larval assays were designed . Unlike the larval photobehaviour assay previously available (Lilly and Carlson, 1990) which tested populations of larvae at a time, these assays test individual larvae and can be used to identify discrete behaviours associated with the larval photoresponse.

The two new single larva assays, the Checker and ON/OFF assays, are relatively quick to perform, making them useful for a large scale screen for mutants. Each quantifies different aspects of the larval visual response, thus whether used in conjunction or separately, they can yield not only complementary data, but also data which can stand alone. Another advantage to these assays, is that testing of single larvae provides the researcher with a tool by which to undertake a mosaic analysis (Hotta and Benzer, 1970). The use of single individuals in this instance is of particular importance as each larva has a distinct pattern of wild type and mutant tissue.

The work described in this thesis shows that *Drosophila* larvae respond to light by modulating their locomotion. In addition, many of the components that have been previously described for adult phototransduction, also have a role in the larval photoresponse as they are able to abolish light perception as measured in these assays. However, these mutations disrupt only subsets of the behaviours associated with the larval perception of light, thus suggesting the existence of light detecting mechanism independent of the main visual pathway described for the adult visual system.

Chapter II

MATERIAL AND METHODS

Fly Stocks

Fly strains were grown at 25°C in 12 hour light/dark cycles on standard medium containing inactivated yeast, sucrose and agar supplemented with fresh active yeast. 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 (gl) The *gl* gene encodes a zinc finger transcription factor required for the development of photoreceptor cells (Moses *et al*, 1989).

gl^{60j}-- a severe allele which contains a 30 kb insertion (Moses *et al*, 1989).

gl^{1}-- moderate allele.

gl^{+}-- contains a wild type *gl* gene in a gl^{60j} background.

***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).

***glass* multimer reporter-*reaper* (pGMR-*rpr*)** This strain contains a fusion vector in which the cell death gene *rpr* is expressed under the control of the *gl* promoter (White *et al*, 1996).

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).

ninaC^{5}-- null mutant which has reduced levels of both the 3.6 and 4.8 kb RNA and leads to 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).

ninaC^{2}-- mutant has reduced levels of the 4.8 kb RNA (Montell and Rubin, 1988).

neither inactivation nor afterpotential E (ninaE) The *ninaE* gene encodes the opsin moiety of the Rh1 rhodopsin and is expressed in the adult photoreceptors R1-R6 (O'Tousa *et al*, 1985) as well as the larval visual system (Zucker *et al*, 1985; Pollack and Benzer, 1988).

ninaE^{17}-- contains a 1.6 kb deletion. 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 in null mutants, 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).

norpA^{P24}-- contains a 28 base pair deletion in the *norpA* gene which produces a premature termination codon (Pearn *et al*, 1996).

norpA^{P12}-- contains a nucleotide substitution in the *norpA* gene which produces a premature termination codon (Pearn *et al*, 1996).

norpA^{+}-- contains a wild type *norpA* gene in a *norpA*^{P24} background (Mckay *et al*, 1995).

sine oculis (so) The *so* gene encodes a homeobox containing protein required for visual system determination (Fischbach and Technau, 1984).

so^{mda}-- Aberrant development of Bolwigs organ (Serikaku and O'Tousa, 1994).

transient receptor potential (trp) The *trp* gene encodes a protein which functions as a cation channel (Hardie and Minke, 1992). *trp* mutants show a reduction in Ca^{2+} currents (Hardie and Minke, 1992; Peretz *et al*, 1994)

trp^{CM}-- A temperature sensitive allele of *trp* it selectively abolishes a class of light sensitive Ca^{2+} channels in photoreceptors (Hardie and Minke, 1992) .

white (w) The product of the *w* gene is normally found in the compound eyes and ocelli of adult flies, adult testes sheaths and larval Malphigian tubules. The *w* gene is believed to encode a membrane associated ATP binding protein that functions to transport pigment

precursors of the ommachrome and pteridine pathways both of which contribute to the red colour of the compound eye (Tearle *et al*, 1989). Flies homozygous for the *w* gene have compound eyes which lack pigmentation and appear white.

yellow, white (yw) The combination of mutant *y* and *w* gene products is often used when tracking chromosomes containing wild type copies of either one or both of these genes. The *y* gene functions in dispersing melanotic pigment in the adult cuticle and in the mouth parts and denticle belts of the larval cuticle. Flies homozygous for *y* possess a yellow body colour. See above for a description of *w*.

***zaroh* (*zaro*)** The *zaro* strain contains a P element insert in the 71F region of the third chromosome. There is an age dependent retinal degeneration in the eyes of adult mutants (DeSousa *et al*, unpublished data)

M2 and **F2** These strains were obtained from a P element screen. The P element contains a viral envelope protein that kills vertebrate cells it is expressed in.

Harvest of synchronized larvae

Adult flies aged from one to seven days were allowed to lay eggs in a fresh food plate (100mm x 15mm; Fisher Scientific) supplemented with vitamin A (Jamisons β carotene, 1.25g/L) and coated with yeast paste. After a minimum of two 2 hour

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 photobehaviour between 84-90 hours AEL. During the freerun test however, a 30 minute egg collection was used. In order not to expose these embryos and larvae to light, no further synchronization was performed.

Photobehaviour assays

Measurements of larval photobehaviour were made on two new assays. These are the Checker and the ON/OFF assays. Both assay consist of a plastic petri dish (100mm x 15mm; Fisher Scientific) containing 15ml of 1% agarose cooled to room temperature. In order to diminish the effect the tensorial stimulus, a circular 1 cm boundary from the plate edge was established.

Manipulation of the larvae prior to the test was conducted using a dark room light (20W lamp with Kodak GBX-2 filter) and 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). Individual larvae were removed, using a moist paintbrush, from the culture dish. Each larva was carefully rinsed with distilled water, transferred using a flathead paintbrush, and placed in a pre-test plate for a period of 1 minute to allow acclimatization

to the agar surface. Each larva was then positioned in the centre of the test plate and allowed to move.

Checker assay Each plate was positioned on a template consisting of 1 cm squares constructed in a checker board manner using black adhesive tape. The dark squares block light while the light squares permit light transmission. Template and dish were positioned on a light box which was modified to emit light only in an 11 cm diametre. During test times, template and dish were lit from below via the light box.

ON/OFF assay Individual plates were placed on a dark background and illuminated from above (20W 'cool white' bulb; Philips in a Rapid Start mechanism; Thomas Lighting) in intermittent 10 second pulses of light and dark.

Plate assay Modified from Lilly and Carlson, 1990. Briefly, approximately 100 larvae were transferred to a plate, positioned on a template which divides the plate into quadrants. Alternating quadrants either block or permit light transmission.

Temperature

Surface temperature recordings were taken in 25 second intervals for 200 seconds during the course of the ON/OFF and Checker assays using a 21X Micrologger (Campbell Scientific Ltd.). Temperature reading in either the Checker assay (light or dark checks), ON/OFF assay or under safelight conditions were 21.5 $\pm$ 0.5 °C.

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) and recorded on videotape (Fuji HQ-120, RCA VCR). In both assays, larvae were recorded either until they reached the 1 cm boundary or until total test time (180 seconds for the Checker assay, 100 seconds for the ON/OFF assay) had elapsed. Data derived for each of the strains was obtained from two to three sets of samples in which ten larvae were tested in each set. In the plate assay, larvae were allowed to migrate for 5 minutes after which the number of larvae in light and dark quadrants was counted and a R.I. derived (Lilly and Carlson, 1990).

Residence time in the dark and light quadrants in the Checker assay were taken using the VCR timer, and started 5 seconds after the larva was placed in the centre dark check. Paths in the ON/OFF assay were first traced from a video monitor (8" x 10" Hitachi 1-chrome) onto acetate sheets and digitized using an Apple OneScanner at 72 dpi. Path length and the angle between path direction before and after the light transitions, were analyzed using public domain NIH Image software (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/) on a Macintosh Performa 5200CD computer. R.I.'s for either assay [(time or path length in dark - time or path length in light)/total time or path length per cycle], were calculated on a per larva basis and a mean average of these individual indices was taken.

The data is depicted as means plus or minus standard error of the mean ($X \pm$ SEM). Transformation of the data was not necessary as variances did not differ significantly (F_{max} test). Analysis of variances (ANOVA's) and Tukey-Kramer multiple comparison tests (α=0.05) were performed on the raw data using SAS-Jmp and Minitab software for Macintosh (Sokal and Rohlf, 1995).

CHAPTER III

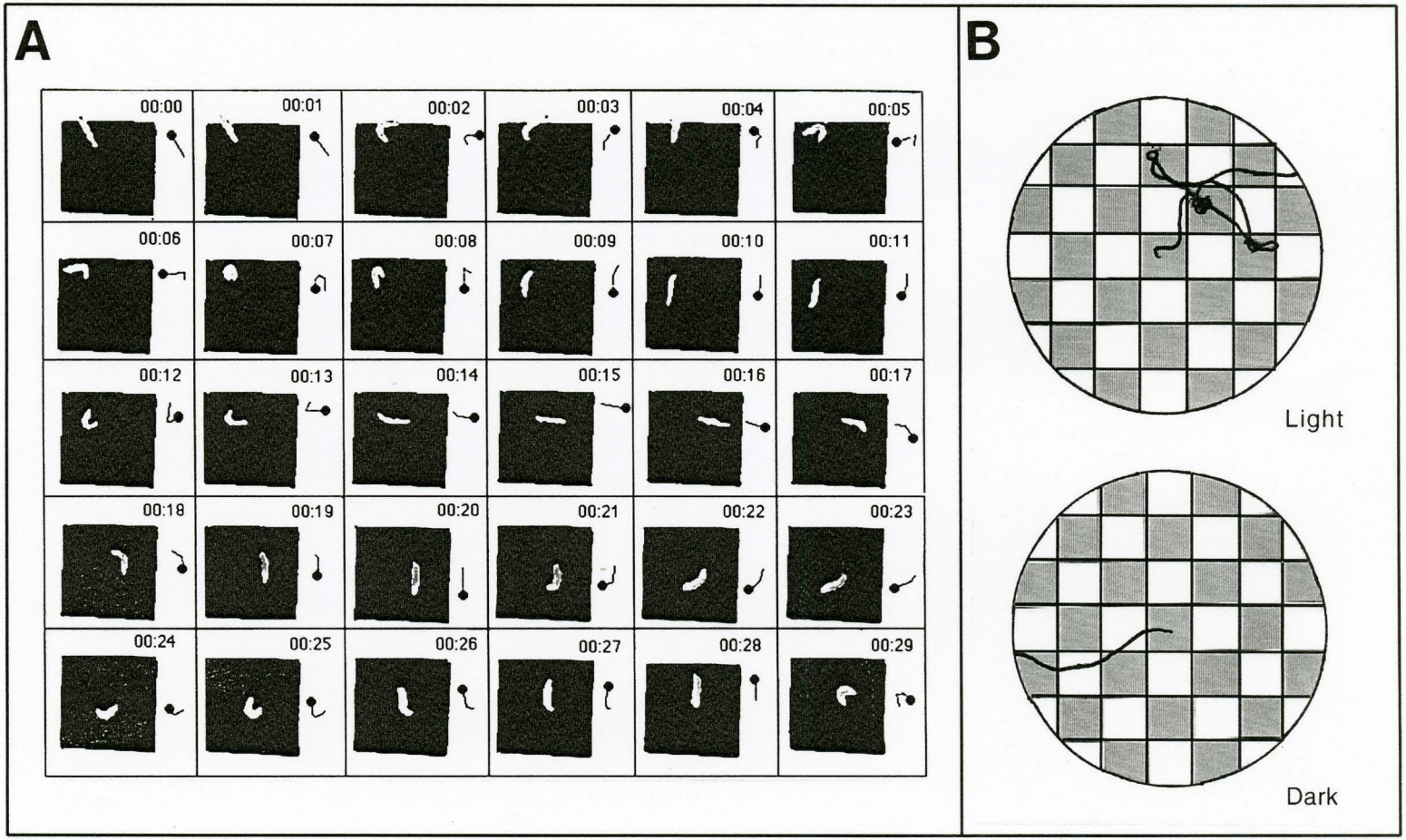
The larval response to light employs many of the same components needed for adult visual system assembly and phototransduction

In *Drosophila*, a number of behavioural assays have been used in genetic screens to identify mutations that disrupt assembly and function of the nervous system. By designing an assay that focuses on finite behaviours it is possible to isolate mutants with specific nervous system defects and then proceed to analyze the genetic lesions that cause them. The use of behaviour assays in genetic screens has been very successful in isolating a number of flies with mutant phenotypes (and finally genes) in the visual system (Benzer, 1967; Pak *et al*, 1969; Harris *et al*, 1976; Hotta and Benzer, 1970; Pak et al, 1970; Gordesky-Gold et al, 1995), the olfactory system (Fuyama, 1976; Rodrigues and Siddiqi, 1978; Aceves-Pina and Quinn, 1979; Siddiqi, 1987; Helfand and Carlson, 1989; Woodard et al, 1989; Lilly and Carlson, 1990; Anholt et al, 1996) and the auditory system (Eberl *et al*, 1997) among others. Presented in this chapter is the Results section of a paper in preparation based on work done in partial fulfillment for my Master's degree. Herein is described the use of two new photoresponse assays, the Checker and ON/OFF assays, to genetically dissect the larval response to light. Further work in the lab will entail using these assays in a genetic screen for larval visual system mutants.

Figure 2. Larval behaviour 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 approached 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. Diagrammatic representations of path taken by a wild type *CS* larva in both test (Light) and control (Dark) conditions.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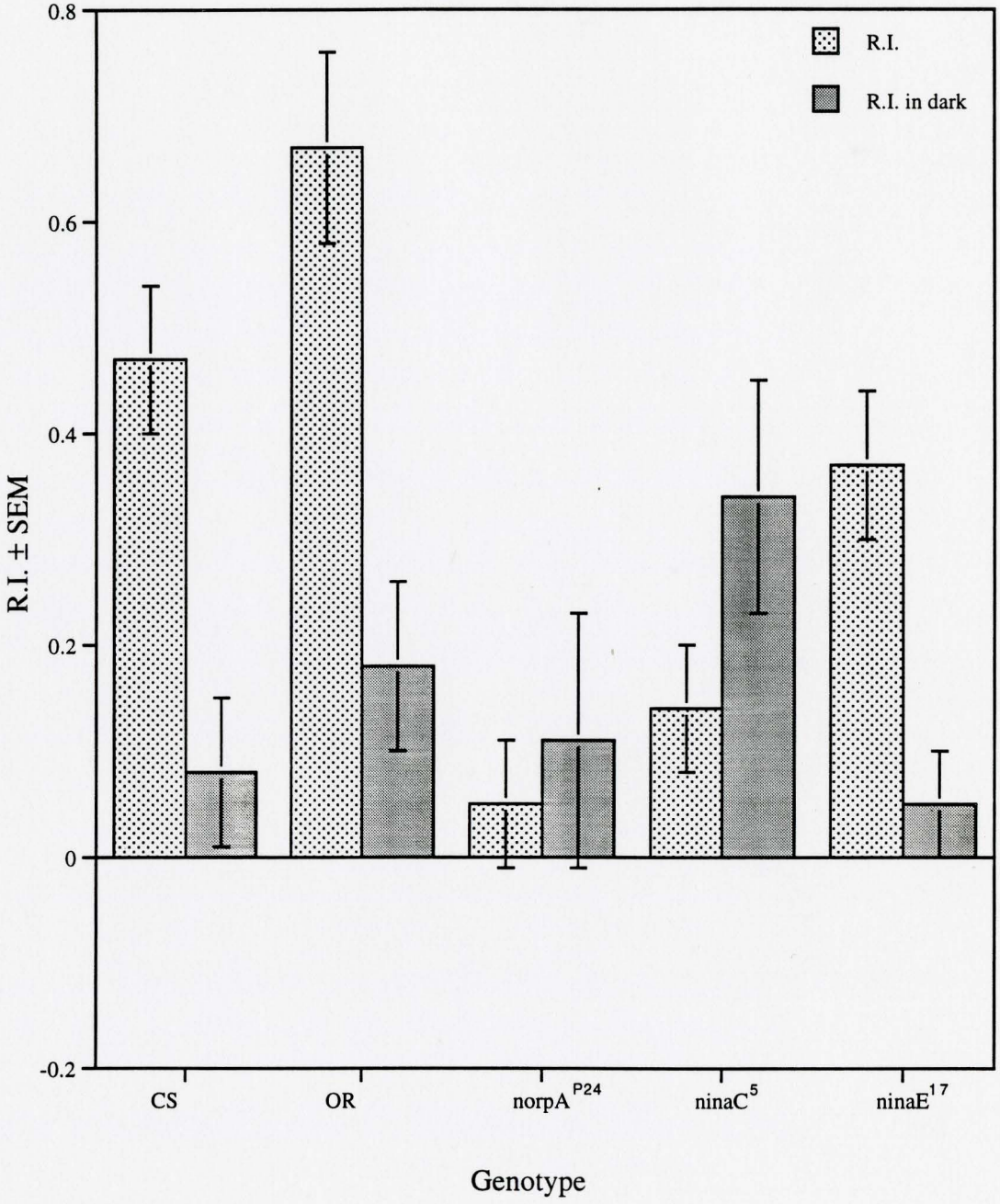
represented by a significant effect of light on the R.I. The R.I.'s obtained with the lights on and off were significantly different in the two wild type strains tested (ANOVA, *CS*-$F_{(1,58)}$=13.45, $p<0.001$, *OR*-$F_{(1,58)}$=15.17, $p<0.001$) indicating an effect of light on larval behaviour (Figure 3).

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 *no-receptor-potential A (norpA), neither inactivation nor afterpotential C (ninaC)* and *neither inactivation nor afterpotential E (ninaE)* genes were tested as described above. These genes encode a phospholipase C, an adult photoreceptor specific cytoskeleton protein and the blue absorbing rhodopsin (Rh1) respectively, all of which are required in the adult fly for visual system function (reviewed by Ranganathan *et al*, 1995).

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 (ANOVA, $norpA^{P24}$-$F_{(1,38)}$=4.2, NS, $ninaC^{5}$-$F_{(1,38)}$=2.65, NS) indicating that mutations in these genes abolish the larval response to light as measured in this assay (Figure 3). In contrast, lack of the blue absorbing rhodopsin Rh1 had no effect on the larval response to light (ANOVA, $ninaE^{17}$-$F_{(1,38)}$=17.94, $p<0.001$) suggesting that in this assay larval response can be mediated by photoreceptors expressing other rhodopsins (Figure 3).

Figure 3. Response in the Checker assay of wild type and larvae with mutations in genes involved in adult phototransduction

Genotypes tested in this assay are wild type strains *Canton-S (CS)* (n=30) and *Oregon-R (OR)* (n=30) as well as the *norpA* (n=30), *ninaC* (n=20) and *ninaE* (n=20) mutant strains which contain mutations in genes involved in phototransduction. Residence time measurements were taken in light during test ($R.I.^{on}$) and in dark ($R.I.^{off}$) conditions. Response to light in this assay is represented by a significant difference between $R.I.^{on}$ and $R.I.^{off}$.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norpA P24
ninaC 5
ninaE 17
Genotype

Light modulates the larval pattern of locomotion

The ON/OFF assay was designed in order to directly estimate the effects of light on locomotion. In this assay, the larva is subjected to intermittent pulses of light and dark (10 seconds each) and its locomotion recorded. Visual inspection of the recorded larval behaviour under the conditions of this assay suggest that distance travelled during the light pulse is considerably shorter. Likewise, head swinging and change of direction of the larval path are apparently triggered by light (Figure 4A,B).

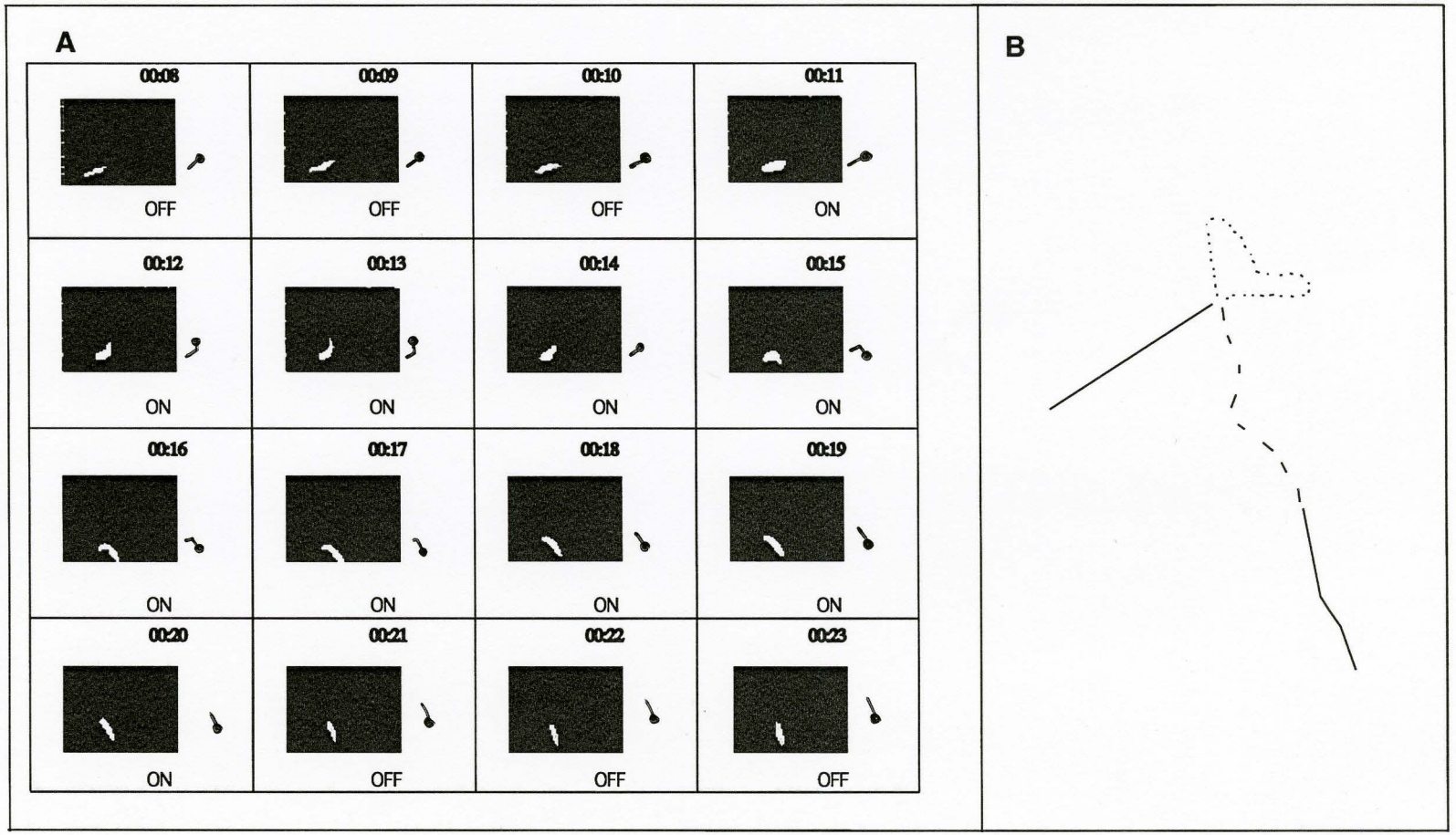
These phenomena were quantified by analyzing the path tracings derived from the recording using an image analysis software (NIH Image). The effect of light on the distance travelled is represented by an R.I. derived from the resulting path length difference between light and dark [(distance travelled in dark)-(distance travelled in light)/ total distance travelled in light and dark]. A R.I. of about 0.3 therefore reflects a 50% reduction in path length when the light is turned on. In order to quantify head swinging behaviour in the two light conditions, path tracings were drawn following the position of the mouth hooks such that head movements as well as the direction of the path were recorded and subsequently counted (Figure 4B).

The wild type strains tested reduce their path lengths when exposed to light as determined by the R.I. obtained (Figure 5). This response was abolished by mutations in genes that disrupt the phototransduction cascade (*norpA*, *ninaC* and *transient receptor potential* (trp^{CM}) but not by mutations in the Rh1 gene (*ninaE*) (ANOVA $F_{(7,181)}=13.42$, $p<0.001$) (Figure 5). The two *ninaC* mutants tested ($ninaC^{5}$ and $ninaC^{2}$), yielded

Figure 4. Larval behaviour in the ON/OFF assay

A. Videotape of a single *CS* larva tested in the ON/OFF assay, was used to generate frame by frame photographs depicting 16 consecutive seconds. To the right of each panel is a schematic diagram of the larva representing the relative position of the head (filled circle) and body (line). The first three frames (seconds 8-10) show a larva immediately prior to a light OFF to ON transition. Lights are turned ON in the 11th second and head swinging is observed (seconds 12-14) followed by a change in direction (seconds 15-18). The final three frames show a larva during lights OFF immediately following the lights ON to OFF transition (seconds 10-21).

B. Line drawing of larval path shown in panel A. The solid lines represent the larval path during the dark pulse (seconds 8-10 and 21-23). The broken line represents the larval path during the light pulse (seconds 11-20). The dotted line depicts the larval head swinging that occurs soon after the lights are turned on. During this time (seconds 12-15) the larva is stationary. This behaviour is followed by a sharp change in the direction of the larval path.


A
00:08
OFF
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OFF
00:11
ON
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ON
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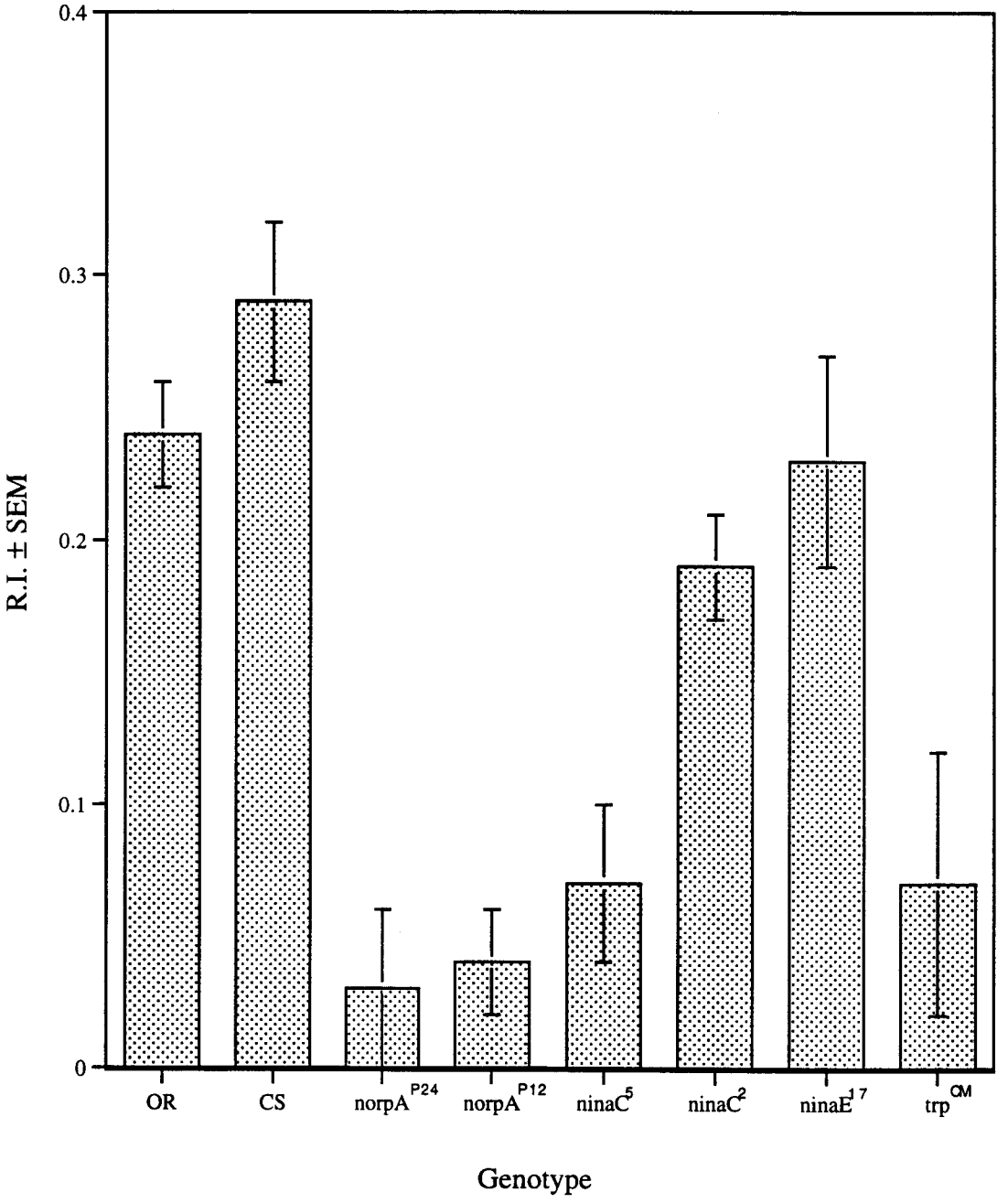
ON
00:14
ON
00:15
ON
00:16
ON
00:17
ON
00:18
ON
00:19
ON
00:20
ON
00:21
OFF
00:22
OFF
00:23
OFF
B

Figure 5. Response in the ON/OFF assay of wild type and larvae with mutations in genes involved in adult phototransduction

A response index (R.I.) was derived per larva and a genotype average calculated. The R.I.'s for the strains are significantly different. Post hoc analysis of paired mean comparisons reveals no differences between the wild type strains (*OR*-n=30, *CS*-n=30) and $ninaE^{17}$ (n=20) but a significant reduction in the larva response to light in the $norpA^{P24}$ (n=30), $norpA^{P12}$ (n=20), $ninaC^{5}$ (n=20), $ninaC^{2}$ (n=19) and trp^{CM} (n=20) mutants.


0.4
0.3
0.2
0.1
0
R.I. ± SEM
OR
CS
norpA P24
norpA P12
ninaC 5
ninaC 2
ninaE 17
trp CM
Genotype

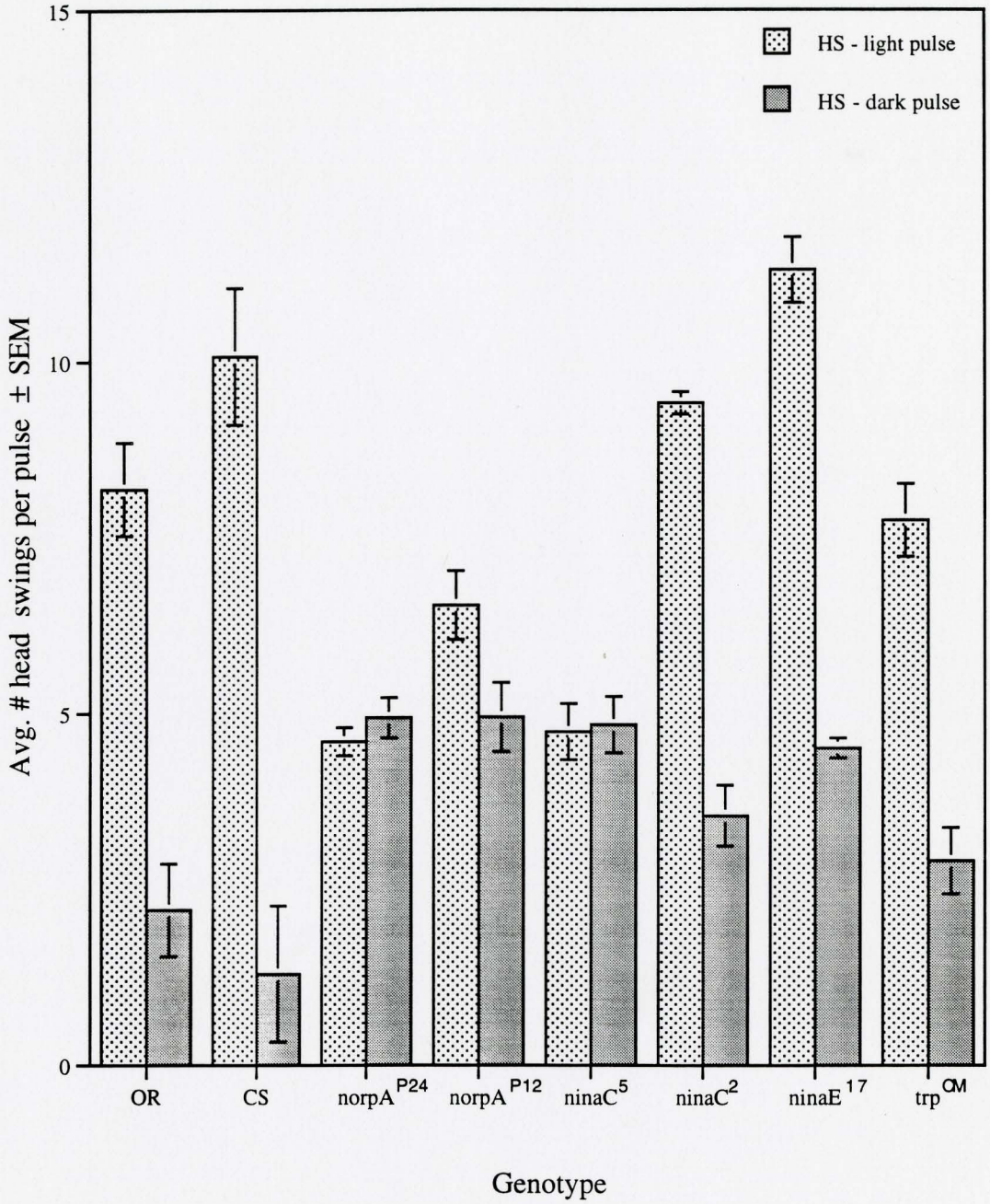
opposite results. *ninaC*^{5} exhibits a mutant and *ninaC*^{2} a wild type larval light response (Figure 5). The *ninaC* gene encodes two adult photoreceptor cell-specific proteins (Montell and Rubin, 1988). One of these a 132 kD protein (p132), is expressed primarily in the cytoplasm. The other, a 174 kD protein (p174) is localized predominantly in the rhabdomere (Porter *et al*, 1992; Hicks and Williams, 1992). While *ninaC*^{5} has reduced levels of both p132 and p174, *ninaC*^{2} has reduced levels of only p174. Therefore, the wild type response seen in *ninaC*^{2} mutant larvae but not *ninaC*^{5} larvae, indicates that p132, not p174, is required for the larval response to light as measured by R.I..

In addition to a reduction in path length during the light pulse, wild type larvae also exhibit increased head swinging in light (ANOVA *CS*-$F_{(1,58)}$=15.69, $p<0.001$, *OR*-$F_{(1,58)}$=20.51, $p<0.001$) (Figure 6). This response was also abolished in the *norpA*^{P24} *norpA*^{P12} and *ninaC*^{5} mutants (ANOVA, *norpA*^{P24}-$F_{(1,58)}$=0.09, NS, *norpA*^{P12}-$F_{(1,38)}$= 2.58, NS, *ninaC*^{5}-$F_{(1,38)}$=0.05, NS) (Figure 6). Again, a wild type response, as measured by head swinging, was seen in *ninaC*^{2} (ANOVA *ninaC*^{2}-$F_{(1,34)}$= 11.53, $p<0.001$) indicating it is p132 that is required for this behaviour also. Wild type responses were also seen in the calcium channel (*trp*) mutants (ANOVA, *trp*^{CM}-$F_{(1,38)}$= 15.22, $p<0.001$) and Rh1 (*ninaE*) mutants (ANOVA, *ninaE*^{17}-$F_{(1,38)}$=30.82, $p<0.001$) (Figure 6).

Taken together, these results suggest that a reduction in path length is due, at least in part, to immobilization of the larva while it swings its head in an apparent search for a dark environment. These responses are carried out by many, but not all, of the same phototransduction cascade components described for the adult visual system. Additionally, these results demonstrate that light induced path length reduction and head

Figure 6. Head swinging behaviour of wild type strains and larvae with mutations in genes involved in adult phototransduction during the ON/OFF assay

Head swings (HS), defined as an abrupt movement of the anterior portion of the larva away from original path choice, were counted in light and dark pulses on a per larva basis and average for each genotype derived. There is a significant increase in head swinging by wild type larvae (*OR*-n=30, *CS*-n=30) during light pulses, relative to that during dark. This difference is abolished in the phototransduction mutants $norpA^{P24}$ (n=30), $norpA^{P12}$ (n=20) and $ninaC^{5}$ (n=20) but not in the $ninaC^{2}$ (n=18), $ninaE^{17}$ (n=20) and trp^{CM} (n=20) mutants.


15
10
5
0
HS - light pulse
HS - dark pulse
Avg. # head swings per pulse ± SEM
OR
CS
norpA P24
norpA P12
ninaC 5
ninaC 2
ninaE 17
trp CM
Genotype

swinging can be mediated by photoreceptors expressing other rhodopsins than the *ninaE* expressing Rh1.

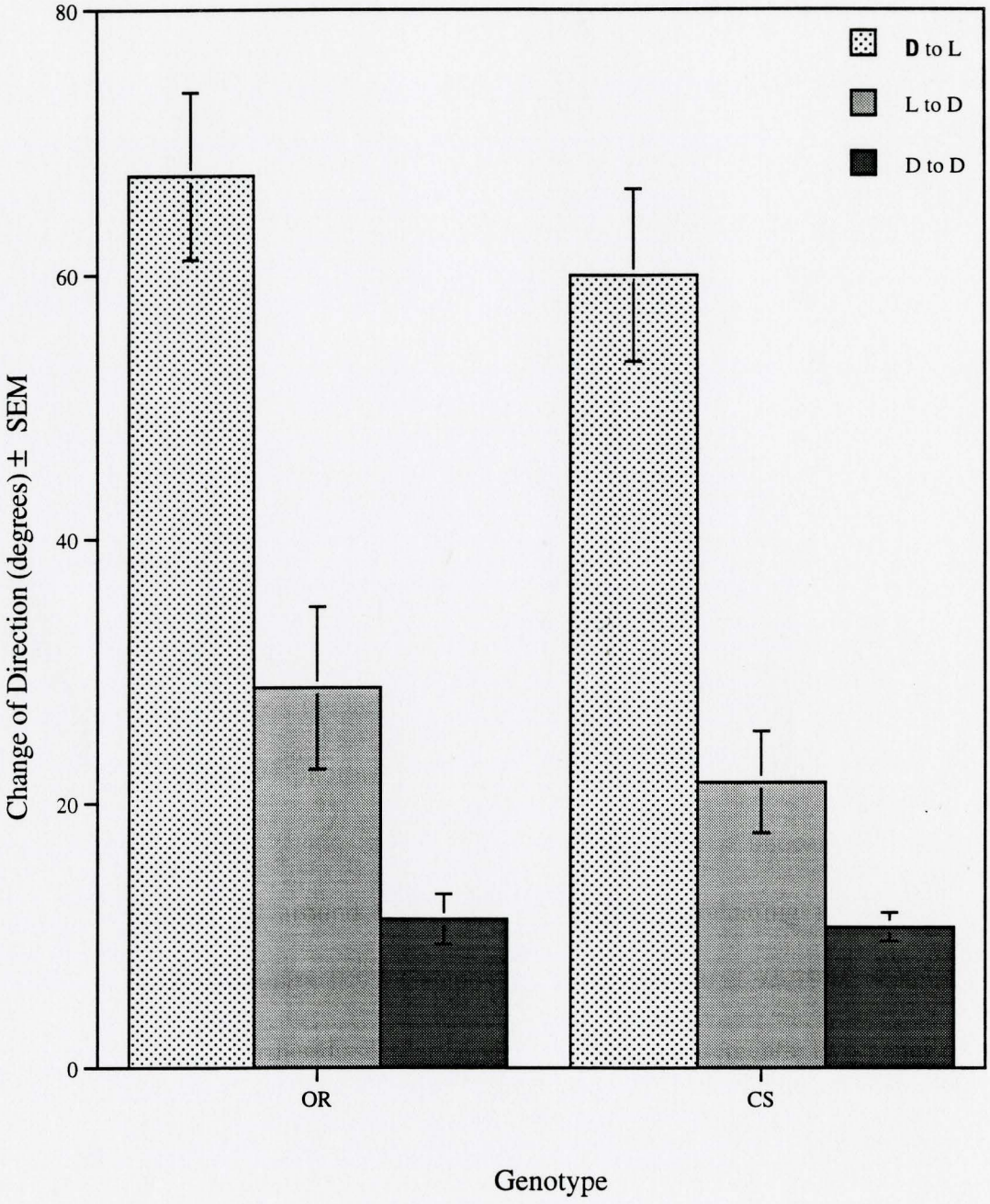
Change of direction in larval path in different light conditions reveals a genetically distinct visual system function

Analysis of path tracings as well as of larval behaviour during the ON/OFF assay suggested that the wild type larva changes direction to a much greater extent when the light is turned on than when it is turned off (Figure 4B). Change of direction in the larval path was quantified by measuring the angle formed by the path tracing at the dark to light and light to dark boundaries. The magnitude of the angle formed by the two paths reflects the magnitude of change in the direction of the larval path at the time of transition. Controls are represented by similar calculations done at 10 second intervals in path tracings derived from recordings performed in the absence of a light stimulus (Figure 7).

Visual inspection of path tracings, suggested that light has a significant effect on path direction. In the wild type strains, direction changes significantly more when the light is turned on (D→L) than when it is turned off (L→D) (ANOVA *CS*-$F_{(1,87)}$=42.49, $p<0.001$, *OR*-$F_{(1,87)}$=33.89, $p<0.001$) (Figure 7). Furthermore, comparison of paired means within genotypes demonstrates that in *OR*, change of direction when the lights are turned off is above that recorded in absence of light transition (D→D). That is, D→L >

Figure 7. Change of direction in wild type strains during the ON/OFF assay

Change of direction (in degrees) was measured at the dark to light (D to L), light to dark (L to D) and in the absence of light (D to D) transitions. *OR* larvae (n=30) display a significant difference between each of the light conditions. *CS* larvae (n=30) display a significant difference between L to D and D to L transition. Although there is a difference in the change of direction between the L to D and D to D transitions, it is not significant.


80
60
40
20
0
D to L
L to D
D to D
Change of Direction (degrees) ± SEM
OR
CS
Genotype

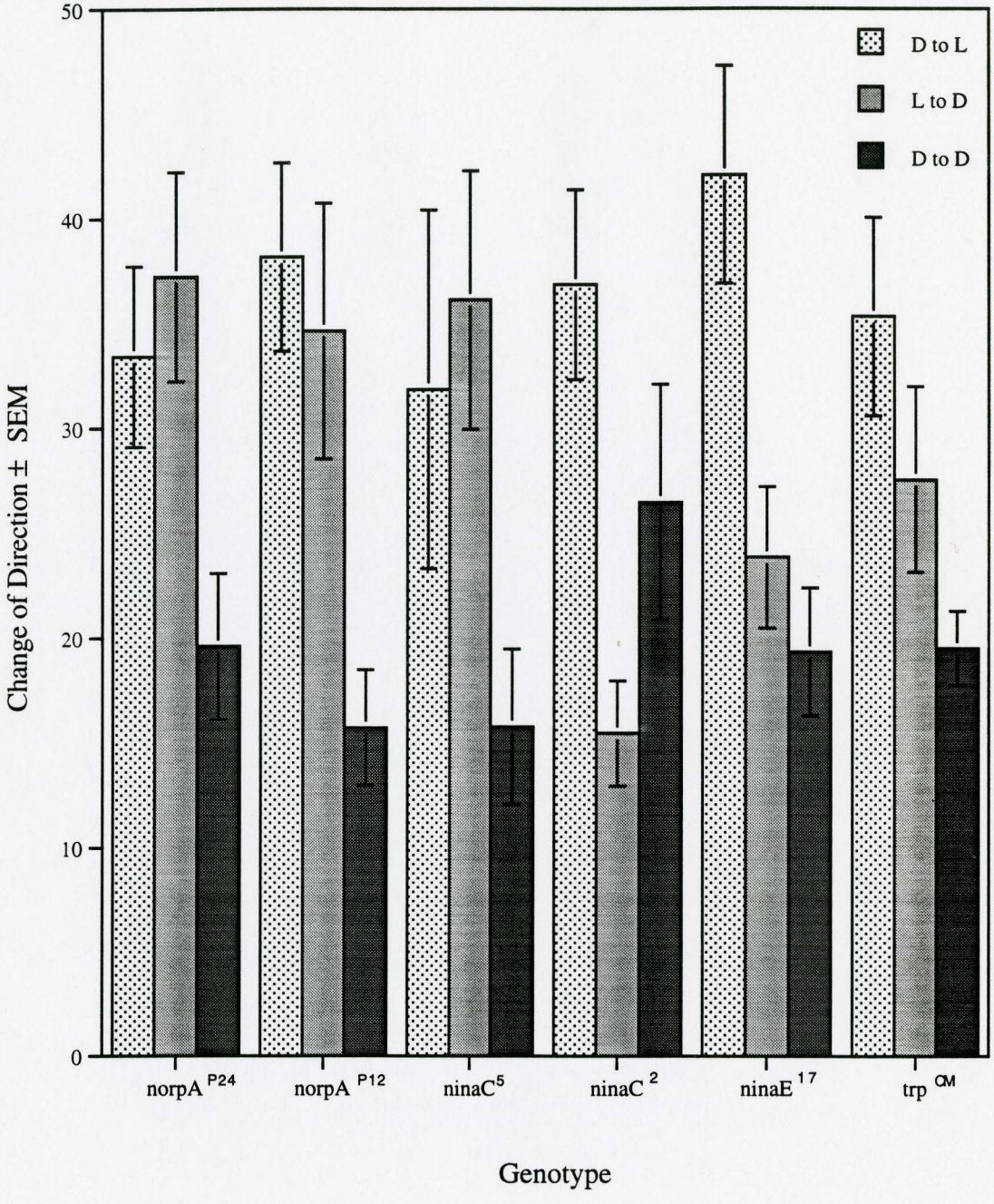
L→D > D→D. While the same phenomena is visible in the other wild type strain (*CS*), it is not statistically significant.

Similar to what is found for the other larval responses to light, mutations in the *norpA* and $ninaC^5$ genes abolish this light induced difference in the amplitude of change of direction. Interestingly, the *norpA* and *ninaC* mutations did not affect the difference between the change of direction found at L→D, and that recorded during the absence of light pulses (ANOVA $norpA^{P24}$-$F_{(2,87)}$=10.12, p<0.001, $norpA^{P12}{}_{(2,57)}$=6.21, p<0.005, $ninaC^5$-$F_{(2,57)}$=5.17, p<0.006) (Figure 8). In contrast to the previous measured responses (R.I. and head swings), $ninaC^2$ does not respond like wild type (Figure 8). While light has a significant effect on direction change (ANOVA $ninaC^2$-$F_{(2,57)}$=5.64, p<0.008) the D→L > L→D > D→D correlation seen in wild type is not exhibited by these larvae. Instead, the only statistically significant comparison is that D→L > L→D. Thus, although in the absence of light transitions the larva changes direction in a manner analogous to both the D→L and L→D transitions, it is still able to respond to stimulus in a wild type fashion.

In contrast, a mutation in the *ninaE* ($ninaE^{17}$) gene reduces the change of direction at the L→D transition to levels indistinguishable from that recorded in the absence of light stimulus. The larvae do exhibit a D→L change of direction that is greater than both the L→D change of direction and direction change in the absence of transitions. The mutation in the *trp* gene abolishes the distinction between the transitions such that only D→L > D→D (ANOVA trp^{CM}-$F_{(2,57)}$=4.48, p<0.02).

Figure 8. Change of direction in strains with mutations in adult phototransduction genes during the ON/OFF assay

Change of direction (in degrees) was measured at the dark to light (D to L), light to dark (L to D) and in the absence of light (D to D) transitions. $norpA^{P24}$ (n=30), $norpA^{P12}$ (n=20) and $ninaC^{5}$ (n=20), exhibit changes of direction at the D to L and L to D transitions which are not different from each other, but which are different from change of direction in the absence of light (D to D). $ninaC^{2}$ (n=20) larvae exhibit a significant difference at the D to L and L to D transitions. Each of these (D to L and L to D) is not different from D to D transitions. $ninaE^{17}$ (n=20) larvae also exhibit a significant difference at the D to L and L to D transitions but the difference between L to D and D to D transitions has been abolished. In the trp^{CM} (n=20) mutant larvae, differential changes in direction are abolished at the light transitions, expect for the difference between D to L and D to D.


50
40
30
20
10
0
Change of Direction ± SEM
D to L
L to D
D to D
norpA P24
norpA P12
ninaC5
ninaC 2
ninaE 17
trp CM
Genotype

These results suggest the existence of a visual system function(s) that distinguishes between lights being turned on (D→L), lights being turned off (L→D) and no light transition. The distinction between lights being turned on and off requires the same phototransduction cascade as that described for R.I. and head swings. That is, it is abolished by mutations in the *norpA*, *ninaC* and *trp* mutants. Rh1-containing photoreceptor function is not required to detect lights being turned on, but is necessary for the larva to distinguish lights being turned off from absence of light. However, mutations in one of the last components in the cascade, *trp*, abolished this distinction. This suggests that the Ca^{2+} channel function encoded by the *trp* gene is essential for larval vision.

Ablation of the Bolwig's organ disrupts only a subset of the larval responses to light

In *D. melanogaster*, the larval visual system (Bolwig's organ) is comprised of two bilateral groups of twelve photoreceptor cells located anteriorly and juxtaposed to the mouth hooks (Bolwig, 1946; Steller *et al*, 1987). These photoreceptors project posteriorly and ventrally around the brain hemispheres, terminating in the optic lobe primordium (Schmucker *et al*, 1997; Campos *et al*, 1995; Green *et al*, 1993; Schmucker *et al*, 1992). In order to further dissect larval visual system requirements, two genes directly involved in visual system specification and development, *so* and *gl*, were studied.

so encodes a homeodomain protein expressed in the optic lobe primordium and Bolwig's organs of embryos, in the developing adult visual system of larvae, and in photoreceptor cells and optic lobes of adults (Cheyette *et al*, 1994; Serikaku and O'Tousa,

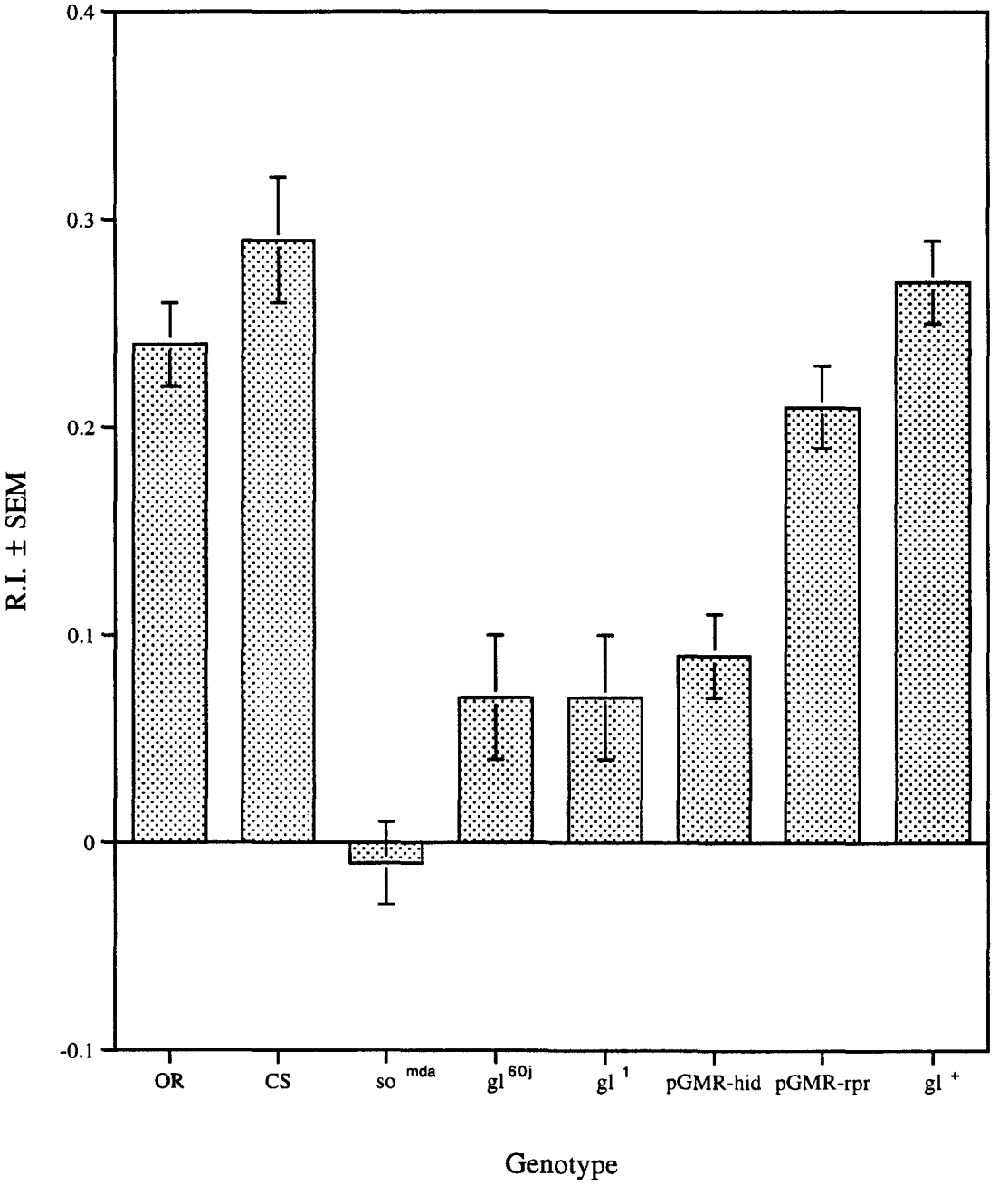
1994). *so* functions include, regulating genes necessary for proper optic lobe invagination and Bolwig's organ formation during embryogenesis (Serikaku and O'Tousa, 1994). The *gl* gene, which encodes a transcription factor essential for photoreceptor development, is expressed in a more spatially restricted manner and acts downstream of *so* (Moses *et al*, 1989; Serikaku and O'Tousa, 1994). *gl* is expressed in the larval and adult photoreceptor neurons as well as in two groups of approximately 21 neurons in each brain hemisphere (Schmucker *et al*, 1992; Moses *et al*, 1989). The effect of *gl* mutations in the development of the *gl* expressing central neurons is not known. This is due to the absence of markers, besides *gl* gene expression itself, that allows the visualization of these neurons.

In order to determine whether the photoreceptors in Bolwig's organ mediate the various responses to light measured in the ON/OFF assay, larvae carrying mutations in the *so* and *gl* gene were assayed. In addition, a *gl* mutant strain displaying wild type adult phenotype, due to the expression of a wild type *gl* gene present in a P element transposon, was tested (Moses *et al*, 1989). Two strains in which a cell death gene (*hid* or *rpr*) is under the control of the *gl* promoter, were similarly analyzed (Grether *et al*, 1995; White *et al*, 1996).

No significant difference between the R.I.'s obtained for the wild type strains and gl^{+} or pGMR-*rpr* was detected (Figure 9). A significant reduction in the R.I. was observed in the so^{mda}, gl^{60j}, gl^{1}, pGMR-*hid* mutant strains (ANOVA $F_{(7,178)}=15.55$, $p<0.001$). Similar results were found when the frequency of head swinging was calculated during light and dark pulses (ANOVA so^{mda}-$F_{(1,38)}= 0.76$, NS, gl^{60j}-$F_{(1,38)}= 0.03$, NS, gl^{1}-$F_{(1,38)}= 0.03$, NS, pGMR-*hid*-$F_{(1,58)}= 0.03$, NS, pGMR-*rpr*-$F_{(1,38)}= 15.33$, $p<0.001$, gl^{+}-

Figure 9. Response in the ON/OFF assay of larvae with mutations in the *so* and *gl* genes.

The R.I. for the strains are significantly different. Post hoc analysis of paired means reveals no difference between the wild type strains (*OR*-n=30, *CS*-n=30) and the pGMR-*rpr* (n=20) and gl^{+} (n=16) strains. A significant reduction is observed in the larval response to light of the so^{mda} (n=20), gl^{60j} (n=20), gl^{1} (n=20) and pGMR-*hid* (n=30) mutants.


0.4
0.3
0.2
0.1
0
-0.1
R.I. ± SEM
OR
CS
so mda
gl 60j
gl 1
pGMR-hid
pGMR-rpr
gl +
Genotype

$F_{(1,30)}$= 9.44, p<0.005) (Figure 10). The significant increase in head swinging frequency during the light pulse displayed by wild type larvae, is abolished by mutations in both *so* and the *gl* genes. This differential head swinging was restored by the gl^{+} containing transposon. Interestingly, while the increase in head swings during the light pulse was abolished in larvae carrying the pGMR-*hid* fusion, larvae containing the pGMR-*rpr* fusion were not affected in this manner.

Ectopic expression of the *hid* gene is sufficient to induce programmed cell death in adult photoreceptors (Grether *et al*, 1995). This expression is also sufficient to ablate the development of the larval photoreceptors labeled by the anti-CHAOPTIN monoclonal antibody 24B10 (Figure 11). Interestingly, in this particular strain, *hid* expression does not affect the viability or differentiation of the *gl*-expressing central brain neurons (Figure 11). Therefore, this strain can be considered as having a specific ablation of the larval photoreceptors identified by *chaoptic* gene expression. In contrast, ectopic expression of a single copy of the *rpr* gene, produces flies with an overall normal eye (White *et al*, 1996). Only when copy number is increased to 4 doses, does ectopic *rpr* expression yields flies with no eyes (White *et al*, 1996). Immunohistochemical studies on the pGMR-*rpr* line were not performed.

Disruption in the development of the larval photoreceptors, caused by *gl* mutations or caused by expression of the *hid* gene, abolished the difference between the magnitude in the change of direction at the D→L and L→D transitions (ANOVA gl^{60j}-$F_{(1,57)}$= 4.42, p<0.02, gl^{1}-$F_{(1,57)}$= 6.23, p<0.005, pGMR-*hid*-$F_{(1,87)}$= 4.57, p<0.01) (Figure 12). However, change of direction in the absence of a light transition is still significantly lower

Figure 10. Head swinging behaviour of larvae with mutations in the *so* and *gl* genes

The head swinging behaviour elicited by light, is abolished in the *gl* mutants (gl^{60j} (n=20), gl^{1} (n=20), pGMR-*hid* (n=30)), which lack larval photoreceptor cells as well as in the *so* (so^{mda} n=20) mutant. pGMR-*rpr* (n=20), which exhibits a less severe adult phenotype than pGMR-*hid*, and the *gl* rescue line, gl^{+} (n=16) display differential head swinging behaviour elicited by light.

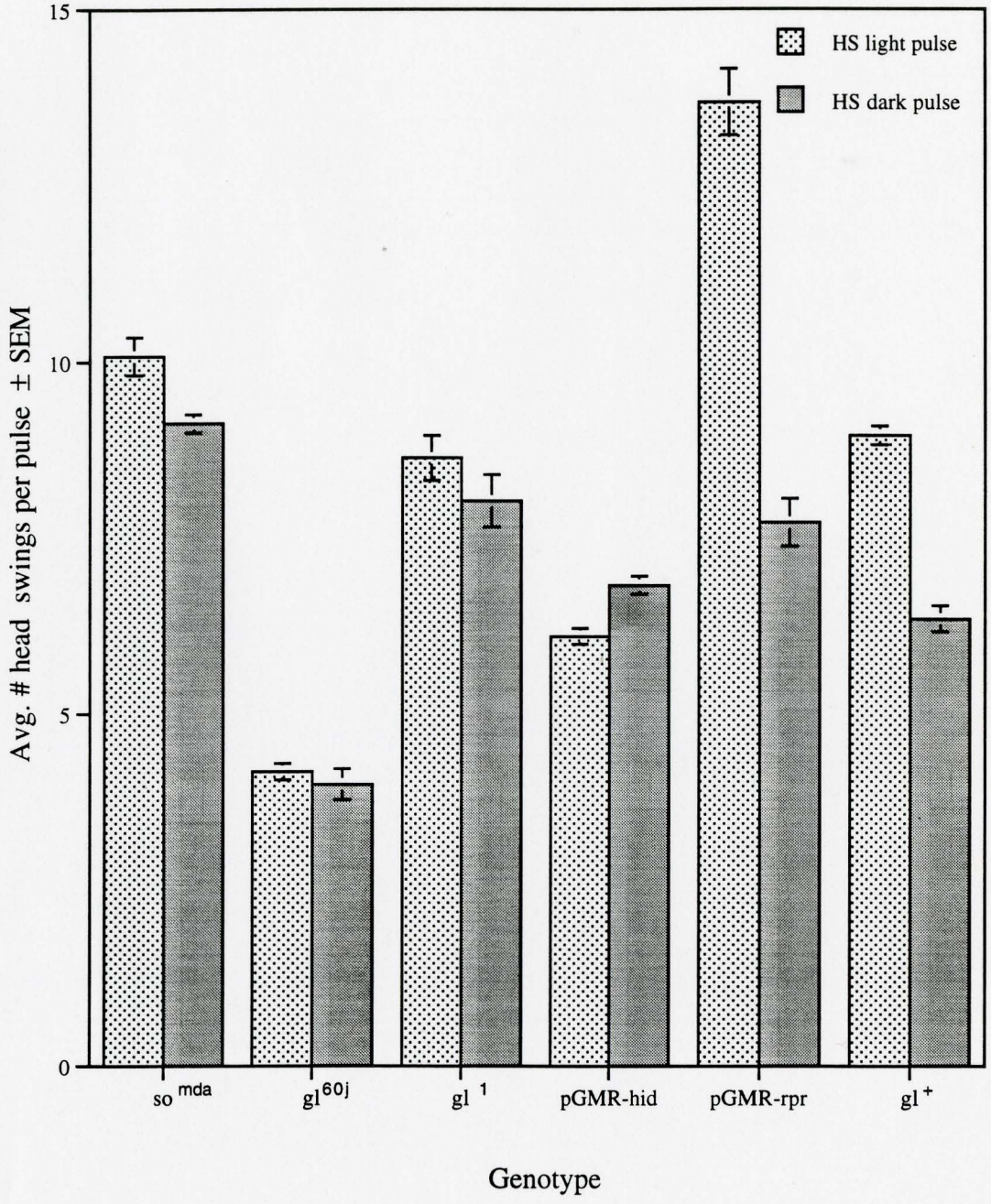

15
10
5
0
HS light pulse
HS dark pulse
Avg. # head swings per pulse ± SEM
so mda
gl 60j
gl 1
pGMR-hid
pGMR-rpr
gl +
Genotype

Figure 11. Staining of photoreceptors in pGMR-*hid* larvae

A. Wild type larval mouth hook and larval photoreceptor cluster stained with 24B10. The arrowhead points at the photoreceptor cluster and the arrow at the nerve bundle from the photoreceptor cluster that projects into the brain.

B. 24B10 staining for larval photoreceptors in a pGMR-*hid* larva showing the absence of larval photoreceptors in this line.

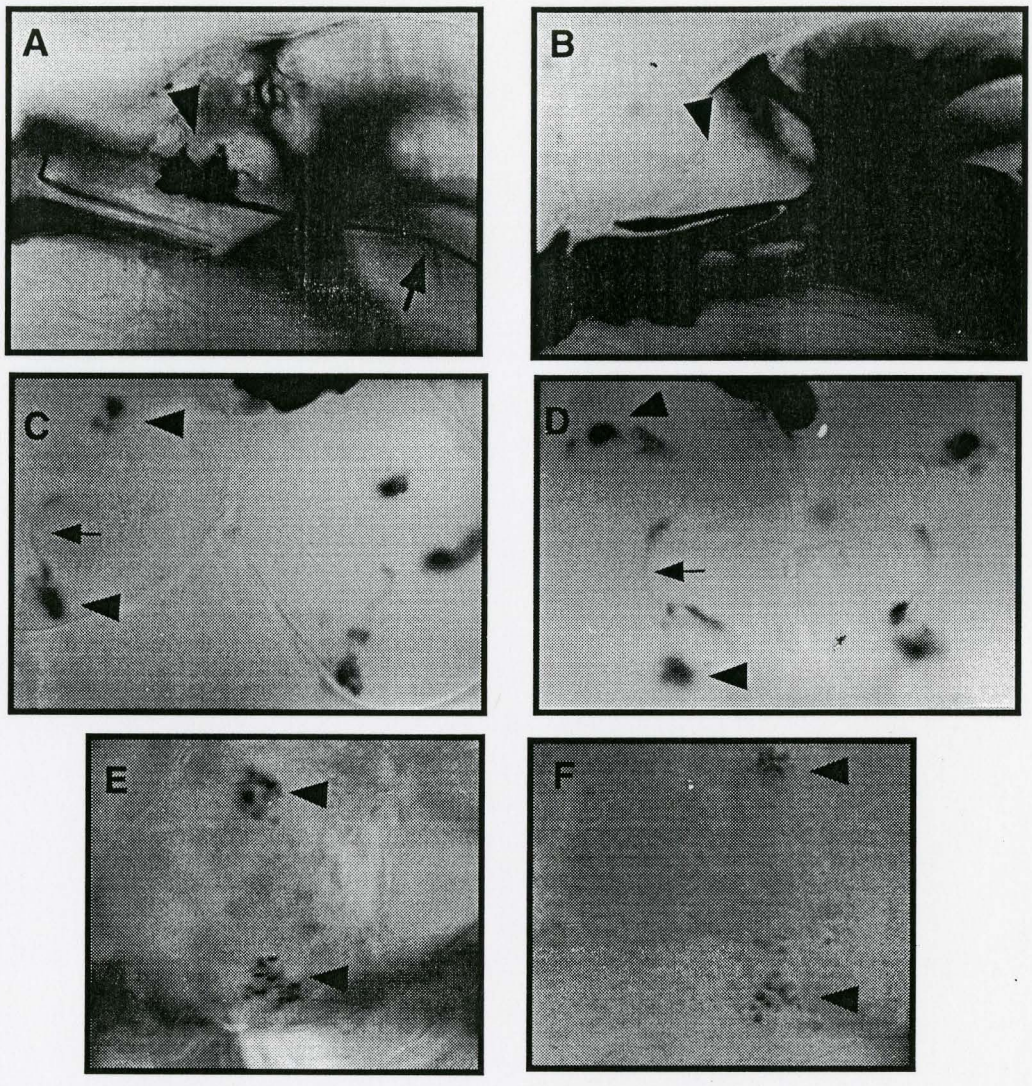
C. Wild type *gl-lacZ* staining pattern in the brain hemispheres. Arrowheads point at the cell cluster that expresses *lacZ* driven by the *gl* promoter. The arrow points at a neuronal aborization that extends from the posterior cluster.

D. *gl-lacZ* staining in a pGMR-*hid* background. The staining pattern does not appear to be very different from the wild type staining pattern in C.

E. *glass* immunoreactivity with a *gl* monoclonal antibody in the right brain hemisphere of a wild type larva

F. Anti-*gl* antibody staining in a pGMR-*hid* larva. The staining pattern does not appear to be very different from the wild type pattern in E.

Work presented in this figure was done by Balaji Iyengar.

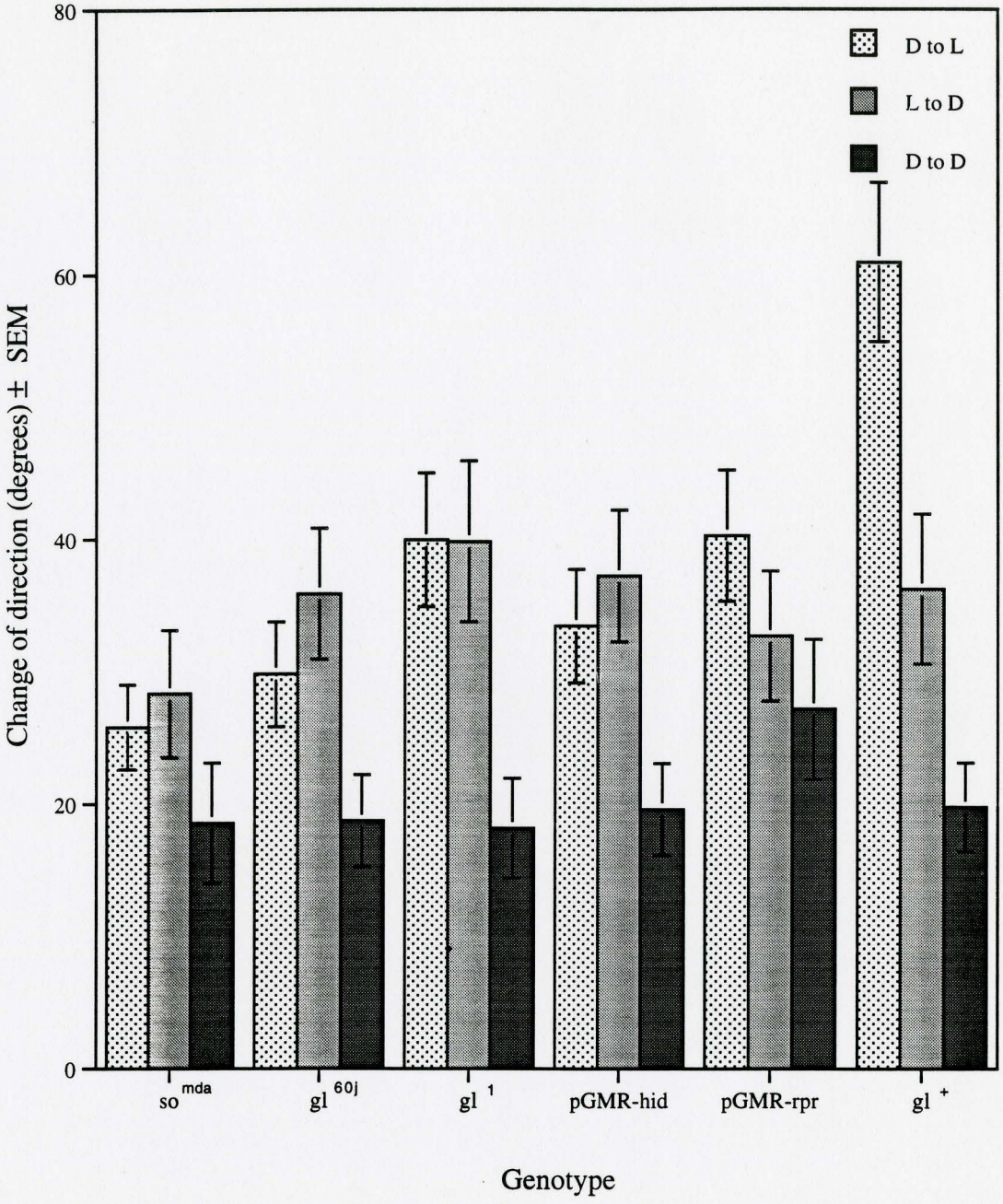

A
B
C
D
E
F

than either of the test conditions (Figure 12). In contrast, a mutation in the *so* gene abolished the difference in magnitude of change of direction at all transitions tested (ANOVA so^{mda}-$F_{(1,57)}$= 1.79, NS). Surprisingly, the pGMR-*rpr* strain also displayed no differential change of direction (ANOVA pGMR-*rpr*-$F_{(1,57)}$= 0.98, NS) (Figure 12).

These results demonstrate that the larval visual function, which is dependent upon a phototransduction cascade similar to that described for the adult stages, requires at least the proper development of the larval photoreceptors. While a mutation in the *so* gene abolishes all responses to light, as measured in this assay, mutations in the *gl* gene appear to disrupt only a subset of these responses. Indeed, mutations in *gl* abolish the larval response to light as measured at or during light transitions. However, *gl* mutant larvae are still able to react to light at a reduced level. These larvae, at the L→D transition, exhibit changes of direction greater than at the D→D transition. Thus, these results demonstrate that larval visual function not dependent on an adult-like phototransduction cascade, is not housed in *gl* dependent neurons but in neurons specified by the homeobox containing transcription factor *so*.

Figure 12. Change of direction of larvae with mutations in the *so* and *gl* genes

Change of direction (in degrees) was measured at the D to L, L to D and D to D transitions. Light has a significant effect on path direction in each of the strains tested, with the exception of pGMR-*rpr* (n=20) and so^{mda} (n=20) in which the presence or absence of light had no effect. The *gl* (gl^{60j}-n=20, gl^{1}-n=20) mutant strains, pGMR-*hid* (n=30) and gl^{+} (n=16) all show no difference between degree of direction change at the light transitions. However, change of direction in the absence of light transitions, is significantly different from either of the test conditions.


80
60
40
20
0
D to L
L to D
D to D
Change of direction (degrees) ± SEM
so mda
gl 60j
gl 1
pGMR-hid
pGMR-rpr
gl +
Genotype

CHAPTER IV

Two new single larval assays test *Drosophila* visual response

During the design and testing stages of any new assay, it must be ensured that the response being studied, in this case the larval visual response, is robust. If the response(s) is not readily reproducible, the chances of the assay yielding interesting and significant information become dramatically diminished. As a result, the single larva assays described herein have been subjected to several types of tests that would allow, not only determination of the reliability of the assays, but also optimization of the test window.

Drosophila melanogaster larvae, like adults, do not respond to red light

To analyze response to light in any organism, one would optimally prefer to work under conditions of complete darkness until the subjected is to be presented with the light stimulus. However in this case, and most cases, this is neither practical nor feasible. Not only is it impossible to prepare the larvae for testing without being able to see them, but larval video recordings can not be done in the absence of illumination.

Previous work in adults, determined that they are unresponsive to light >610 nm, very little has been done to test if the same is true in larvae (Dowse and Ringo, 1989). Sawin *et al.* (1995) reported that larvae do not respond to light ≥ 610 nm. In

order to confirm this finding, plate assays (Lilly and Carlson, 1990) were performed in which light was transmitted through a Kodak GBX-2 filter, blocking out light < 610 nm. Thus, the larvae were exposed to two alternating red and dark quadrants. Under these circumstances the R.I.'s obtained were < 0.1, whereas the same larvae tested with white light yielded R.I.'s > 0.6 (Table 1).

Since the larvae were able to respond in the plate assay when illuminated by white light, this ruled out the possibility that their lack of response when illuminated by red light was a false negative. These results therefore, indicate that red light can be used as an alternative to dark conditions when testing larval visual response.

Response Index is robust over the 100 seconds of the ON/OFF assay

Once it had been established that larvae did not respond to red light it became a priority to set limits for the assays themselves. Like in any test, it was necessary to set a reasonable time limit for testing larval visual response in the ON/OFF assay. Unlike the plate assay described by Lilly and Carlson which derived a R.I. after a 5 minute test, the times selected for the ON/OFF and Checker assays is considerably shorter. There are several reasons for this decreased test time:

- as these tests are to be used in a mutant screen, it is preferable that test time be kept relatively short

- once the larva finds the plate edge, it has a tendency to remain there.

Table 1. Response Indices of larvae tested in the plate assay

R.I.'s of larvae tested in the plate assay in white light (without GBX-2 filter) and in red light (>610 nm) (with GBX-2 filter) conditions. Approximately 100 larvae were tested in each trial.

Genotype	Trial	R.I. with GBX-2 filter	R.I. without GBX-2 filter
OR	1	-0.04	0.64
	2	0.04	0.82
	3	-0.03	0.73
CS	1	-0.03	0.62
	2	0.08	0.80
	3	0.10	0.62

As a result, 100 seconds was selected as the time limit for this assay. R.I.'s derived for wild type larvae over the course of the 100 seconds did not vary (ANOVA $F_{(4,87)}$=1.77, NS) (Figure 13). In each case a R.I. was calculated over 20 seconds in the assay, such that each R.I. encompassed a light and dark pulse. In the Checker assay, a time limit of 180 seconds was set. The rationale behind this limit was based more on visual observations than on numbers. It was observed during the initial testing period in the Checker assay, that even larvae that were responding to light, as measured by the assay, left the test arena in 180 seconds or less. In both assays in addition to a time limit, a circular 1 cm boundary from the plate edge was established. Thus measurements in either assay were taken until either the larvae crossed the test boundary, or the time limit had elapsed.

Larvae can respond to light of a variety of wavelengths

In addition to red light tests, different light sources with slightly different spectrums were analyzed. These were: incandescent (450 - 1200 nm, with peaks in far red), 'daylite' (400 - 650 nm with peaks at 400, 450 and 550 nm) and 'cool white' (400-650 nm with peaks at 440 and 560 nm) lights. None of these light sources produced significantly different R.I.'s in the ON/OFF assay, in either of the wild types tested (ANOVA $F_{(5,40)}$= 1.47, NS) (Figure 14). Results of these experiments indicate that larval visual response is robust over a variety of wavelengths. All behavioural measurements were done using 'cool white' light.

Figure 13. Response Index in the ON/OFF assay over 100 seconds

R.I. was calculated over the course of the ON/OFF assay in 20 second segments such that each segment consists of distance travelled in one light and one dark pulse. R.I. did not vary significantly over the 100 second time limit of the ON/OFF assay.

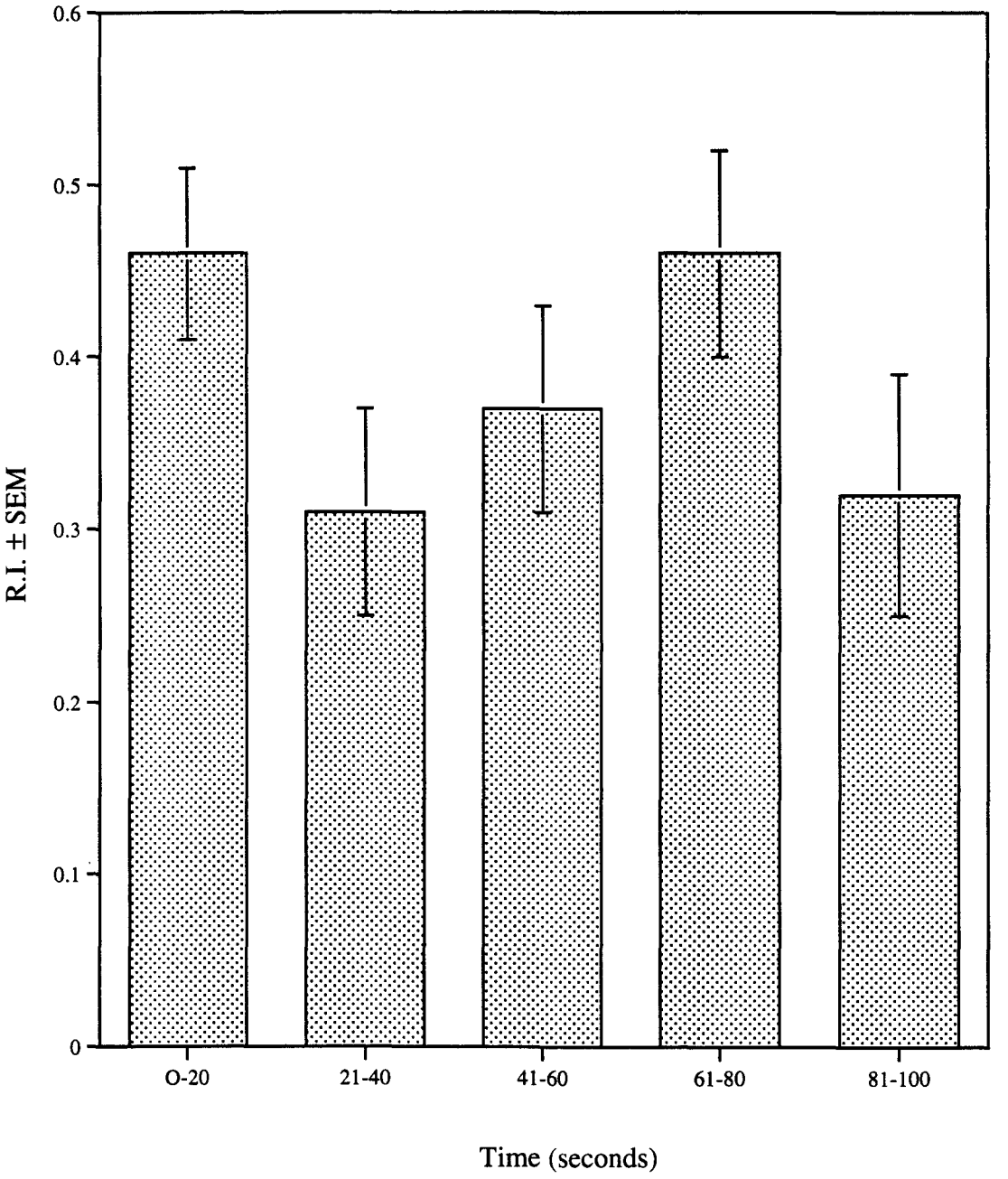
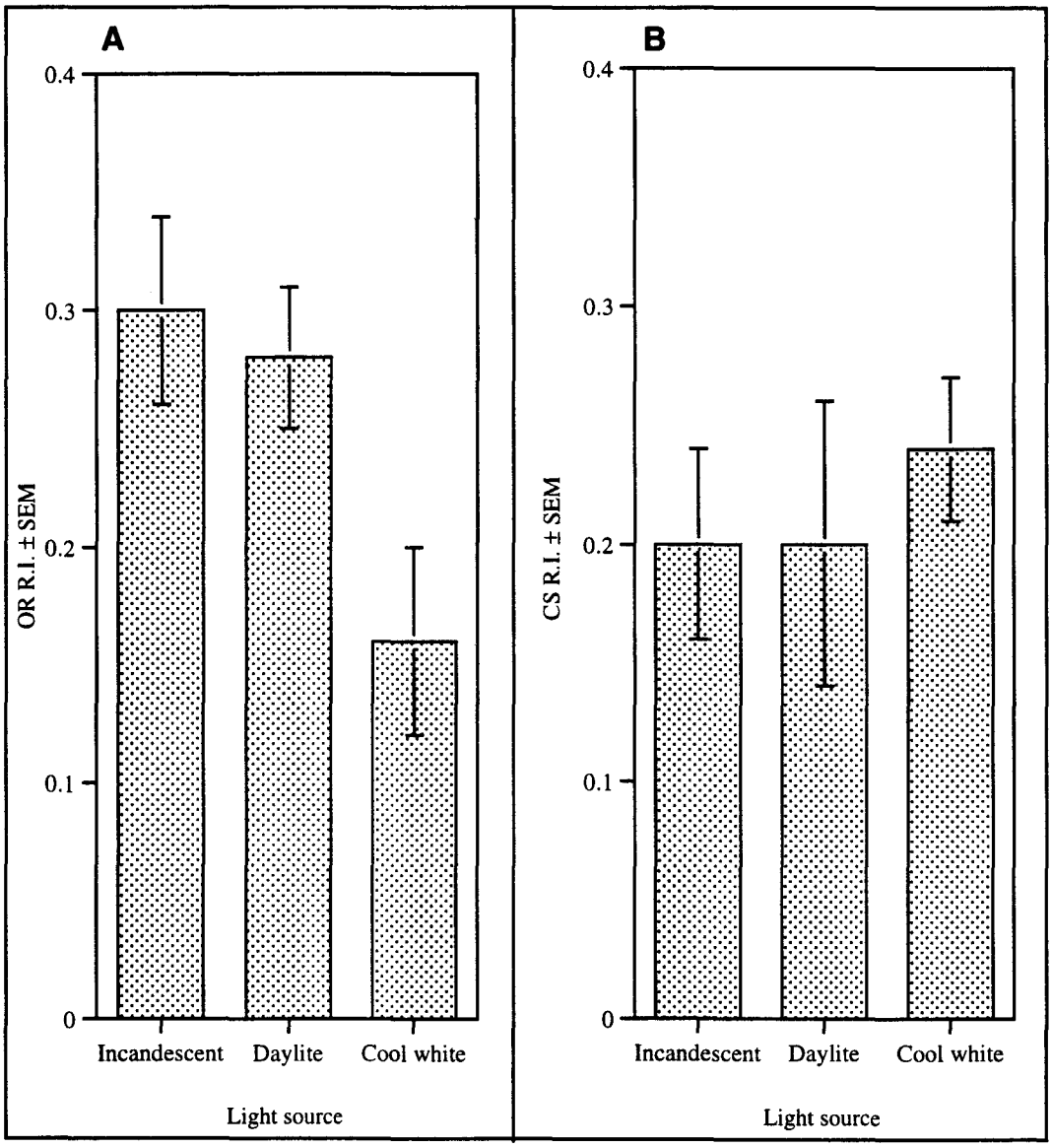

0.6
0.5
0.4
0.3
0.2
0.1
0
R.I. ± SEM
O-20
21-40
41-60
61-80
81-100
Time (seconds)

Figure 14. Response in the ON/OFF assay for two wild type strains tested in different light conditions

Larvae were tested for visual response in three different light sources with slightly different spectrums. These were incandescent (400 - 1200 nm and peaks in the far red of the spectrum), daylite (400 - 650 nm with peaks at 400, 440 and 550 nm) and cool white (400-650 nm with peaks at 440 and 560 nm). The R.I.'s for either *OR* (A) or *CS* (B), using cool white, incandescent and daylite light sources were not significantly different.


A
0.4
0.3
0.2
0.1
0
OR R.I. ± SEM
Incandescent
Daylite
Cool white
Light source
B
0.4
0.3
0.2
0.1
0
CS R.I. ± SEM
Incandescent
Daylite
Cool white
Light source

Response indices were maintained throughout an 18 hour test window containing both light and dark segments of one circadian cycle

In both the Checker and ON/OFF assays, larvae were initially tested 84 - 86 hours after egg lay (AEL). This limited not only the number of trials that could be done in any one day but also imposed strict limitations on the experimenter. Thus, in order to both expand the testing window and examine what effects, if any, circadian time had on larval photoreception and response, a series of experiments in the ON/OFF assay were performed during 24 hours of third instar from 90-98 hours AEL.

Behaviour tests were performed every six hours starting at 80 and ending at 98 hours AEL, therefore four sets of experiments per cycle were conducted; 80, 86, 92 and 98 hours AEL. Under standard laboratory conditions, lights are turned on at 10:00 hours and off at 22:00 hours. This was termed the 'regular' cycle. The reciprocal experiments involved the light being turned on at 22:00 hours, and off at 10:00 hours. This was termed the 'reverse' cycle . As a result, in the 'regular' cycle 80 and 86 hours were in subjective night while 92 and 98 hours in subjective day. The opposite was true in the reverse cycle such that 80 and 86 hours were in subjective day and 92 and 98 hours in subjective night (Figure 15). R.I.'s derived for any of these times points were not significantly different from each other (ANOVA, $F_{(7,102)}=1.13$, NS) (Figure 16). Distances travelled in 30 seconds (Figure 17) were found to be significantly different from each other (ANOVA, $F_{(7,102)}=3.27$, $p<0.003$) although, paired mean comparisons indicated that only 80 hours 'regular' was different from 92 and 98 hours 'reverse'.

Figure 15. Diagrammatic representation of 12 hour Light/Dark cycles in the ON/OFF assay

A set of experiments were done which tested larval response to light in the ON/OFF assay at six hour intervals from 80 - 98 hours AEL. Testing was staggered such that, two of the time points took place during lights on of a 12 hour light-dark (L/D), and two during light off. This experiment was repeated twice more, once in a 12 hour L/D cycle opposite ("reverse") to that normally employed ("regular"), and once in complete darkness ("freerun").

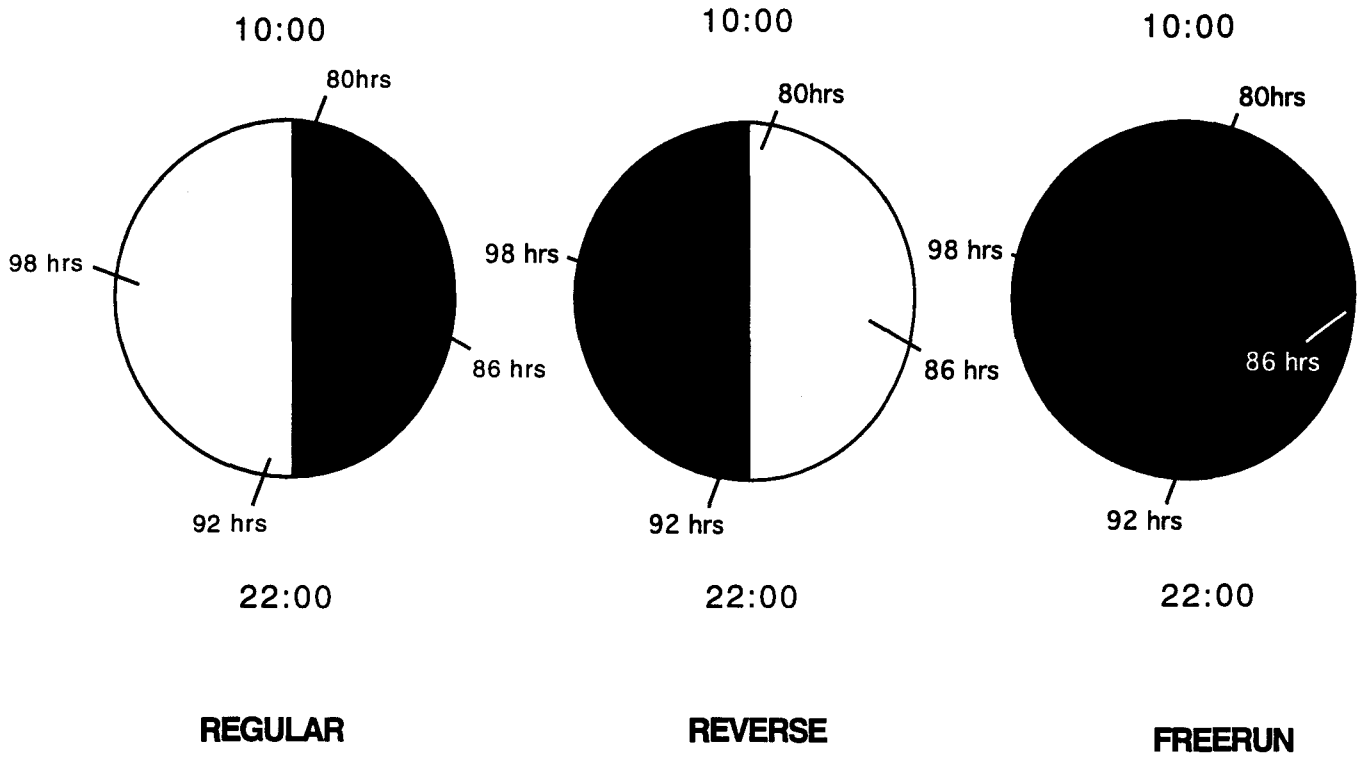

10:00
80hrs
98 hrs
86 hrs
92 hrs
22:00
REGULAR
10:00
80hrs
98 hrs
86 hrs
92 hrs
22:00
REVERSE
10:00
80hrs
98 hrs
86 hrs
92 hrs
22:00
FREERUN

Figure 16. Response in the ON/OFF assay of third instar larvae tested during an 18 hour time window including light and dark segments of one circadian cycle

A R.I. was calculated at each of four time points in the ON/OFF assay (80, 86, 92 and 98 hours) for wild type *CS* larvae. Two sets of experiments were done, one in the "regular" 12 hour L/D cycle (lights turn on at 10:00) (80 hrs-n=15, 86 hrs-n=15, 92 hrs-n=15, 98 hrs-n=15) and one in the "reverse" 12 hour L/D cycle (lights turn on at 22:00) (80 hrs-n=15, 86 hrs-n=5, 92 hrs-n=15, 98 hrs-n=15). R.I.'s taken at any of these time points are not significantly different from each other.

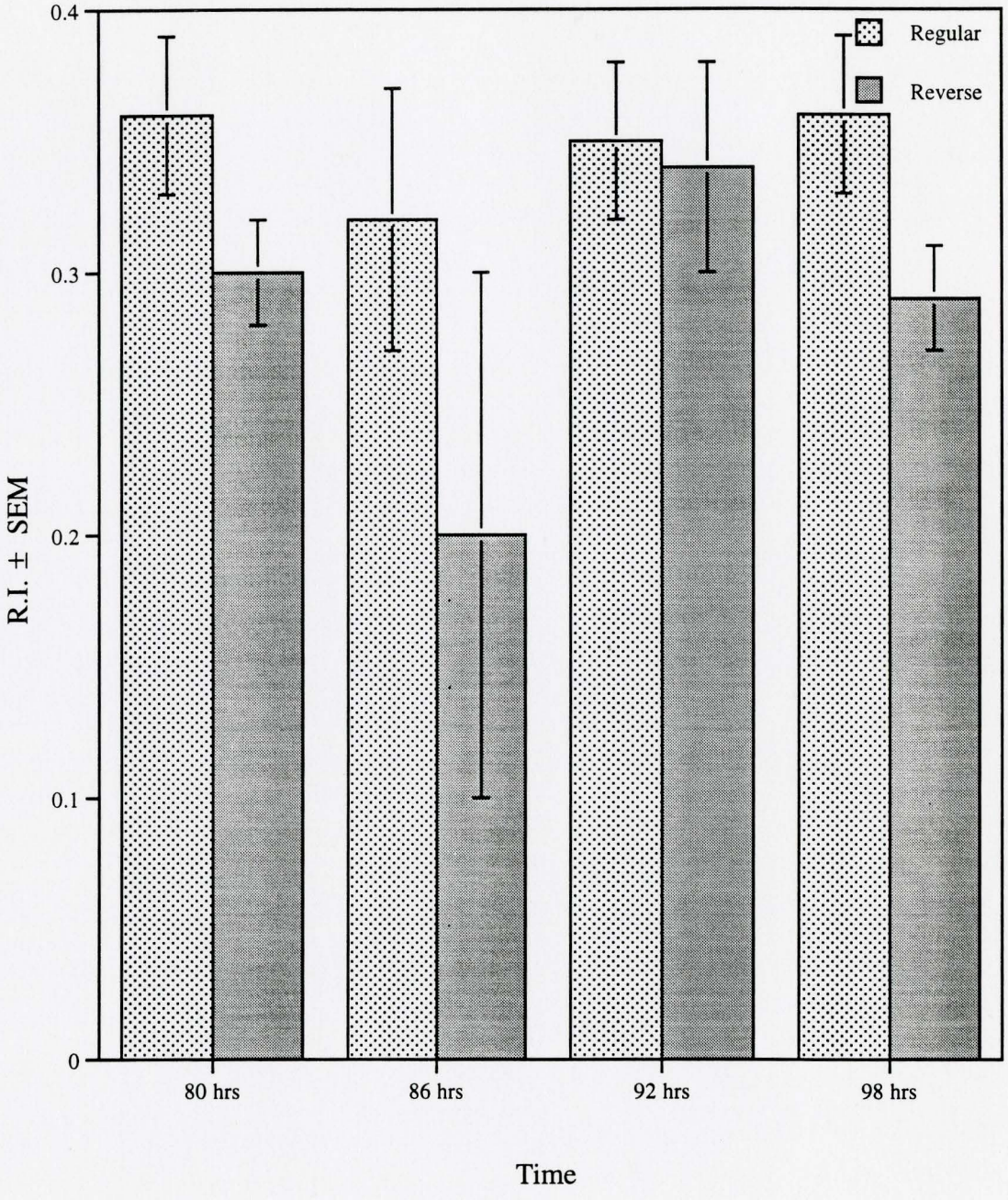
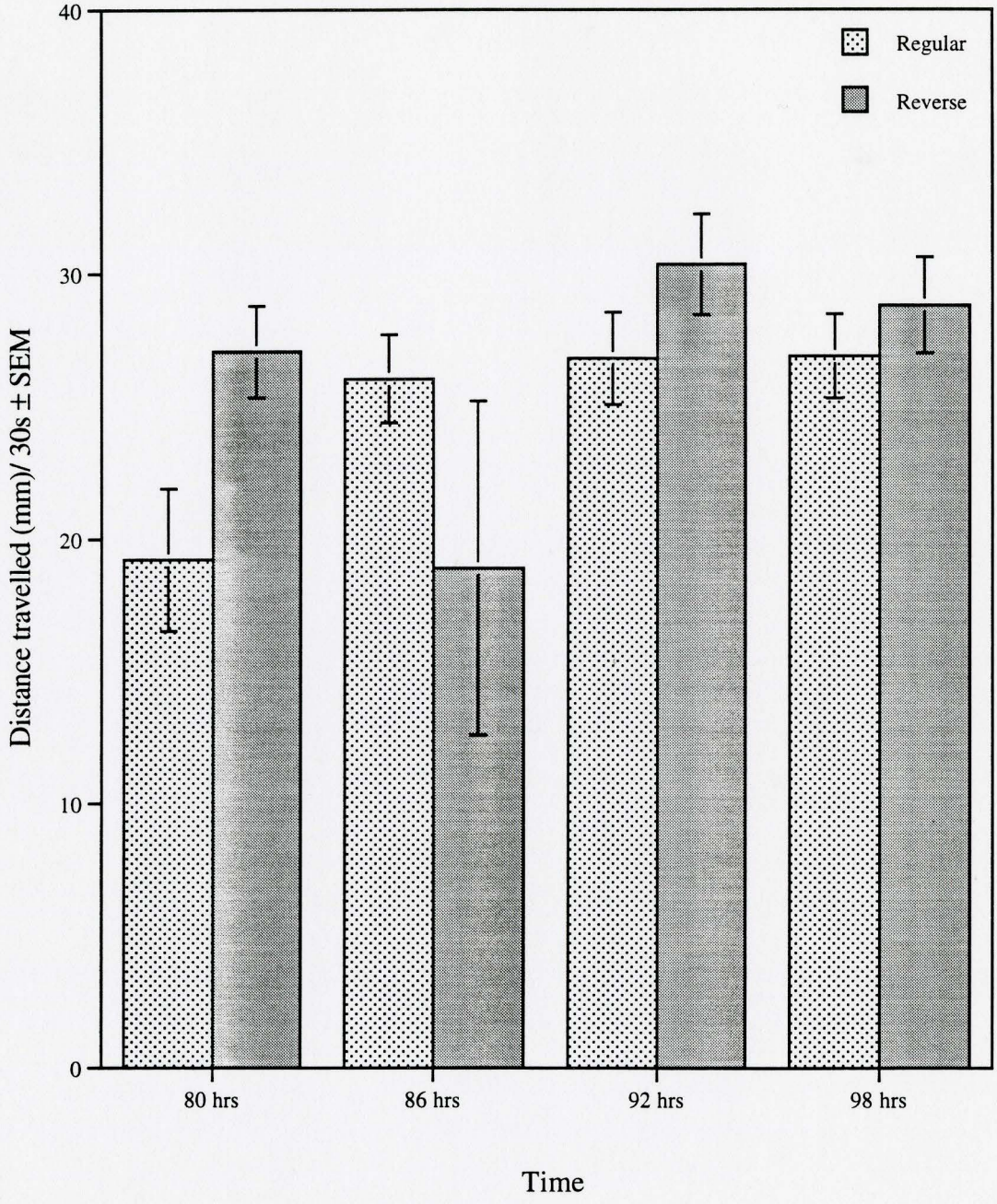

0.4
0.3
0.2
0.1
0
R.I. ± SEM
Regular
Reverse
80 hrs
86 hrs
92 hrs
98 hrs
Time

Figure 17. Distance travelled in 30 seconds by third instar larvae tested during an 18 hour time window including light and dark segments of one circadian cycle

Distance travelled in 30 seconds was measured at each of four time points in the ON/OFF assay (80, 86, 92 and 98 hours AEL) for wild type CS larvae. Two sets of experiments were conducted, one in the "regular" 12 hour L/D cycle (lights turn on at 10:00) (80 hrs-n=15, 86 hrs-n=15, 92 hrs-n=15, 98 hrs-n=15) and one in the "reverse" 12 hour L/D cycle (lights turn on at 22:00) (80 hrs-n=15, 86 hrs-n=5, 92 hrs-n=15, 98 hrs-n=15). Distance travelled at the time points measured are significantly different from each other. Post hoc comparison of paired means reveal that only distance travelled at 80 hours "regular" is different from distance travelled at 92 and 98 hours "reverse". All other distances are equal.


40
30
20
10
0
Regular
Reverse
Distance travelled (mm)/ 30s ± SEM
80 hrs
86 hrs
92 hrs
98 hrs
Time

Freerun conditions affect R.I. but not locomotion

An additional cycle of experiments were performed, in which the larvae were reared in complete dark conditions (absence of light/dark cycles). Under these conditions, the larvae tested displayed significantly reduced R.I.'s from the previous two tests (regular and reverse) (ANOVA, $F_{(11,158)}=2.21$, $p<0.016$) (Figure 18). Distance travelled however, was not different from results obtained in regular and reverse cycles.

***glass* mutant larvae display decreased locomotory scores**

It has previously been reported that on non nutritive substrates larval locomotion increases and turning decreases, when compared to measurements done on nutritive substrates (Green *et al*, 1983; Troncoso *et al*, 1987). Under 'dark' conditions wild type larvae (*CS, OR*) travel 19-26 mm in the course of 30 seconds (Figure 19). During this time little turning behaviour is observed and the larva has a propensity to travel in a straight line. However, once testing of known adult visual system mutants began (see Materials and Methods), it quickly became apparent that larvae with severely decreased locomotion would be difficult to test. Since many of the components quantified in the ON/OFF and Checker assays require that the larva be able to move, distance travelled in 30 seconds was measured on a non nutritive substrate in the absence of light. This measurement allows a distinction between larvae that appear to respond to light but really

Figure 18. Response in the ON/OFF assay during an 18 hour time window including light and dark segments of one circadian cycle as well as a freerun trial

In addition to the four sets of time points in the "regular" and "reverse" 12 hour L/D cycles, one set of experiments was done during the same time points in the absence of light transitions (80 hrs-n=15, 86 hrs-n=15, 92 hrs-n=15, 98 hrs-n=15). R.I.'s taken during these time points are significantly different from each other. Post hoc analysis of paired mean comparisons indicate that R.I.'s measured during the first two time points in the freerun (80 and 86 hours), are significantly different from R.I.'s measured at any other time point in either the "regular", "reverse" or freerun cycles.

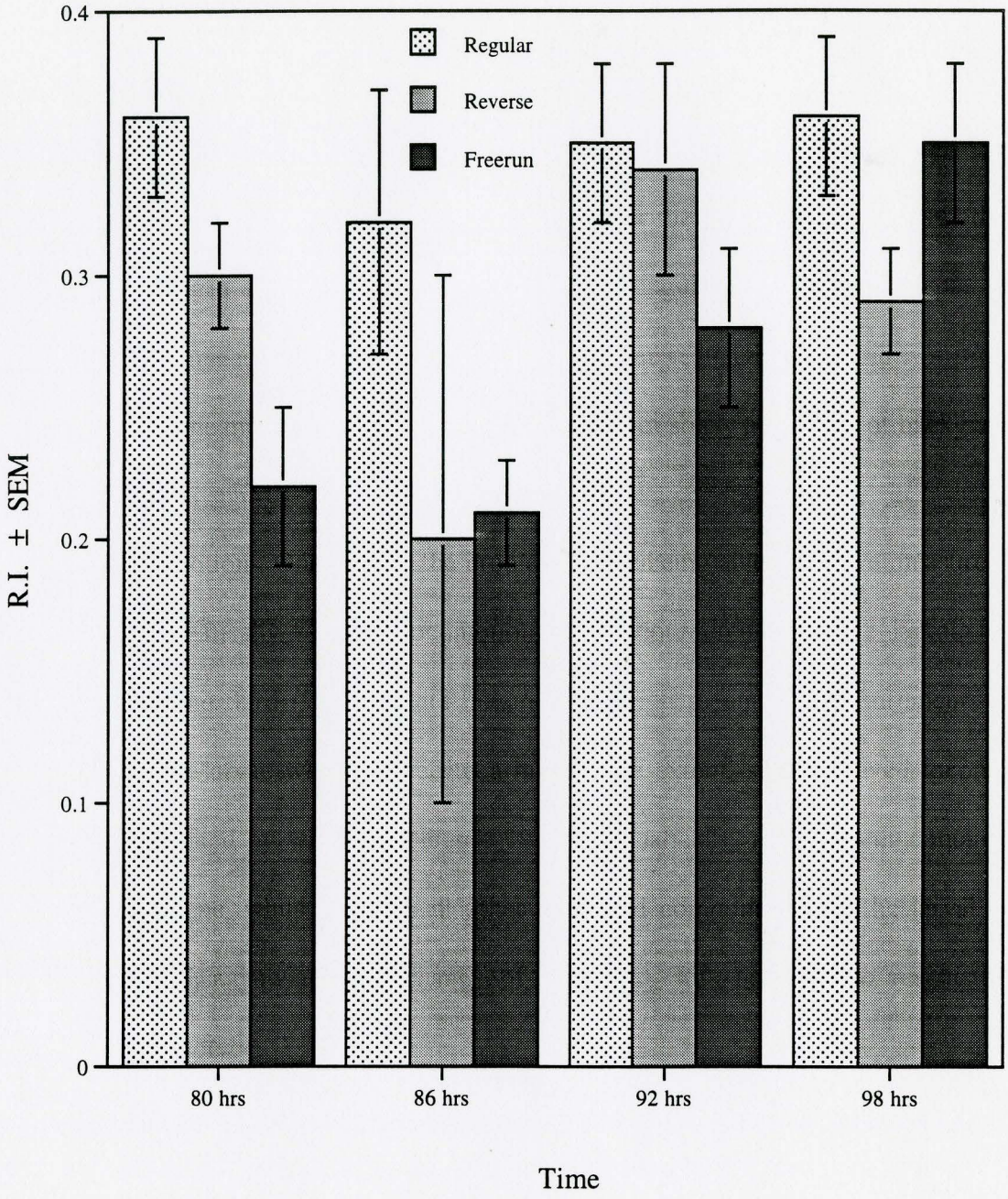
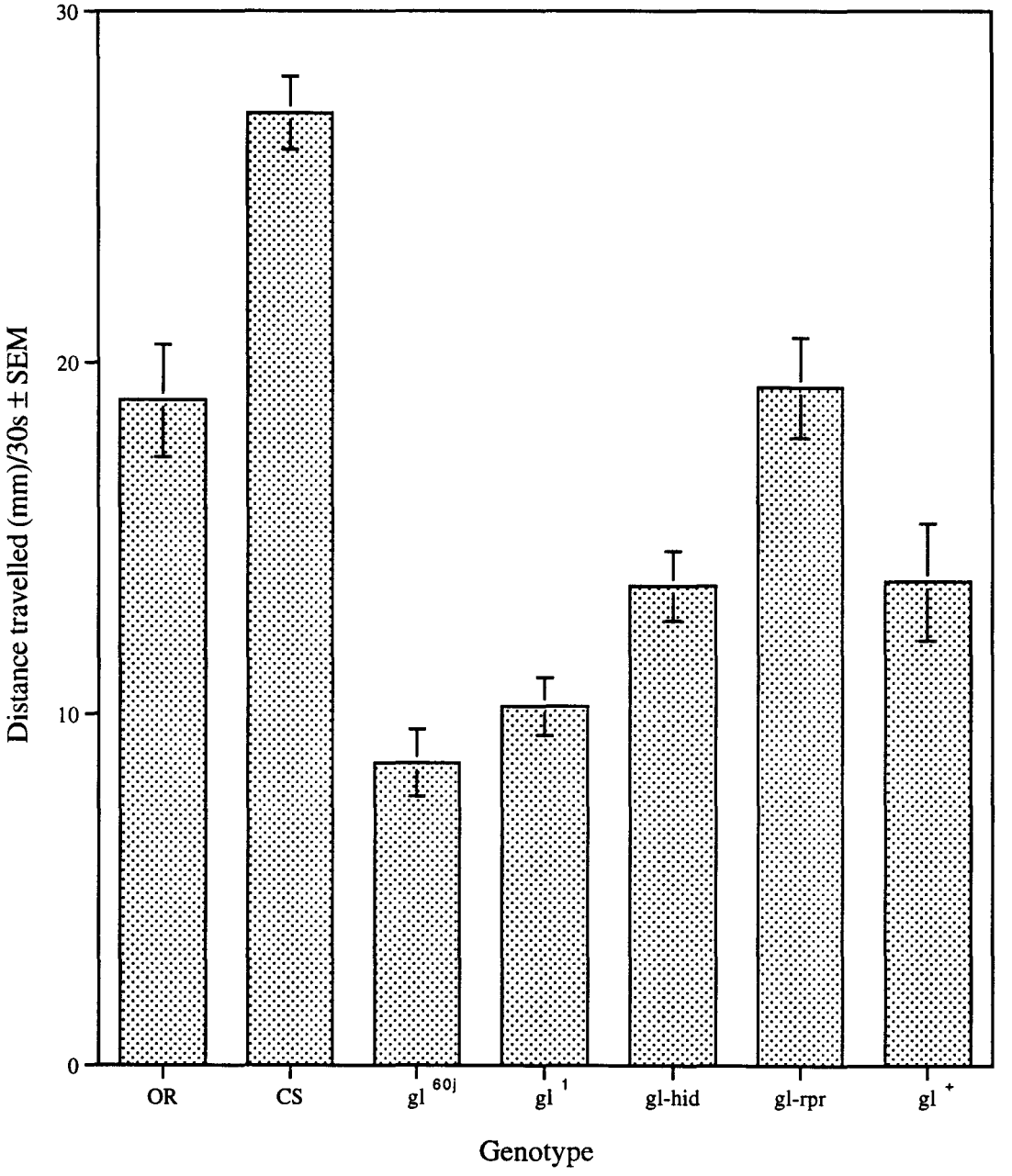

0.4
0.3
0.2
0.1
0
R.I. ± SEM
Regular
Reverse
Freerun
80 hrs
86 hrs
92 hrs
98 hrs
Time

Figure 19. Distance travelled in 30 seconds by wild type and larvae with mutations in the *gl* gene, pGMR-*hid*, pGMR-*rpr* and gl^{+}

Distance travelled in 30 seconds was measured in the ON/OFF assay in wild type (OR-n=30, CS-n=30) and *gl* (gl^{60j}-n=20, gl^{1}-n=20) mutant larvae. The *gl* mutant strains, pGMR-*hid* (n=30) and the *gl* rescue strain, gl^{+} (n=16), all exhibit decreased locomotion when compared to wild type. Distance travelled by pGMR-*rpr* (n=20) is indistinguishable from wild type.


30
20
10
0
Distance travelled (mm)/30s ± SEM
OR
CS
gl 60j
gl 1
gl-hid
gl-rpr
gl +
Genotype

had locomotory defects, and a larvae which respond to light. As a result, locomotion was considered as a gauge of the overall "wellness" of the larva.

Although no adult locomotory phenotype has been reported in strains with mutations in the *gl* gene, gl^{60j} mutant larvae consistently had severely reduced locomotion. Such was the reduction that no measurements in the ON/OFF assay could be taken. As the larvae did not move they were never tested in the Checker assay.

To test the possibility that this locomotory phenotype could be due to background, a recombinant gl^{60j} strain was tested. This recombinant was essentially the same as the original gl^{60j} strain tested, except that the left arm of the third chromosome (the *gl* gene is on the right arm) had been recombined away using the FLP-FRT system. Locomotion improved in these larvae to an extent where measurements in the ON/OFF assay could be taken in all larvae tested. However, locomotion was still low when compared to wild type (Figure 19). As genetic information contained on the right arm of the third chromosome in the original strain, was still present in the recombinant strain, the presence of genetic modifiers contributing to this larval locomotory phenotype could not be ruled out (Nuzhdin et al, 1997). Further analysis of other *gl* mutant alleles was done to further test this possibility.

In total two strains with mutations in the *gl* gene, two strains expressing the cell death genes *hid* and *rpr* under control of the *gl* promoter, and a *gl* rescue line (gl^{+}) were tested. In each instance, distance travelled by any of these strains was 9-14 mm, significantly less than travelled by wild type (ANOVA, $F_{(6,159)}= 27.76$, $p<0.001$) (Figure 19). The only exception was the pGMR-*rpr* strain. This strain contains a transgene which

expresses the cell death gene *rpr* under control of the *gl* promoter. In this instance, distance travelled was indistinguishable from wild type. The lack of the decreased locomotory phenotype associate with the rest of the *gl* mutant lines, could be attributed to the fact that pGMR-*rpr* must be present in copy numbers of three or greater before its effects on the adult eye phenotype are visible (White *et al*, 1996). This increased copy number is presumably also required in order to uncover the larval phenotype as both, R.I. and head swings, in addition to locomotion, are indistinguishable from wild type. Since the locomotory phenotype persisted, this indicated that reduced locomotion in probably associated with the mutations in the *gl* gene itself and not solely a product of background.

Whereas, pGMR-*rpr* exhibited normal levels of locomotion, gl^{+} displayed decreased locomotion. Once again, the problem of not being able to derive measurements because of dramatically reduced locomotion was encountered. Of the larvae tested, approximately one third (7 of 23) did not move enough to enable measurements to be taken. Even the larvae which moved to a measurable extent, still displayed locomotion below that exhibited by wild type (Figure 19). Enigmatically, a P element containing a functioning *gl* gene, while rescuing all other measured components of the larval visual response (R.I., head swings and change of direction), was not able to rescue the *gl* locomotory phenotype.

At the same time however, tests involving a *norpA* rescue line ($norpA^{+}$) were proceeding. These results indicated that this strain also had decreased locomotion when compared to both wild type and the mutant background ($norpA^{P24}$), into which the $norpA^{+}$ P element transposon was inserted. Thus, the possibility arose that, in addition to

endogenous locomotory phenotypes, insertion of P elements themselves could somehow deleteriously affect larval locomotion.

Strains containing P element insertions also display a reduction in locomotion

In order to test whether P elements do indeed have an effect on larval locomotion, a number of P element lines were analyzed. These P element lines were chosen at random from strains already present in the lab. In addition to the *norpA*$^{+}$ strain, which expresses a wild type *norpA* transcript (subtype I) under control of the *ninaE* promoter, three other P element containing lines were tested. One line, *zaro*, expresses the *lacZ* gene under control of the acetylcholine transferase promoter. This was then cloned into a mini-*w* containing vector, thus linking the gene of interest to a marker gene (*w*), which manifests itself distinctly in transformants (Klemenz et al, 1987). The other two lines, M2 and F2, express a viral toxin under the control of a *heat shock* promoter (*hsp*). In this instance, heat shocking the flies at 37 °C induces expression of the toxin. In addition to the P element lines tested, two strains commonly used as recipient fly stocks for P element mediated transformation , *w* and *yw*, were analyzed.

All lines tested, including *yw*, *w* and the P element insert lines (*norpA*$^{+}$, *gl*$^{+}$, *zaro*, *M2* and *F2*) showed R.I.'s in the ON/OFF assay indistinguishable from wild type (ANOVA, $F_{(8,183)}=10.44$, $p<0.001$) (Figure 20). However, with the exception of *w*, their locomotory scores are significantly different from wild type (ANOVA, $F_{(8,183)}=43.00$, $p<0.001$) (Figure 21). Although locomotion of *w* mutant larvae is indistinguishable from

Figure 20. Response of wild type and five strains containing P element insertions

R.I.'s were derived for five independent P element containing strains. Two strains contain wild type copies of a gene, which is then inserted into a mutant background in an attempt to rescue a mutant phenotype (*norpA*$^{+}$ (n=15), *gl*$^{+}$ (n=16)). The other three strains were created as a result of a P element mutagenesis screen such that they disrupt gene function in the gene where they were randomly inserted (*zaro*-n=20, M2-n=20, F2-n=20). In addition, two strains normally used as hosts for P element transformation (*w* (n=20) and *yw* (n=20)) were tested. The only strain which exhibits a decreased R.I. is *norpA*$^{+}$. All other strains exhibit R.I.'s indistinguishable from wild type (*OR*-n=30, *CS*-n=30).

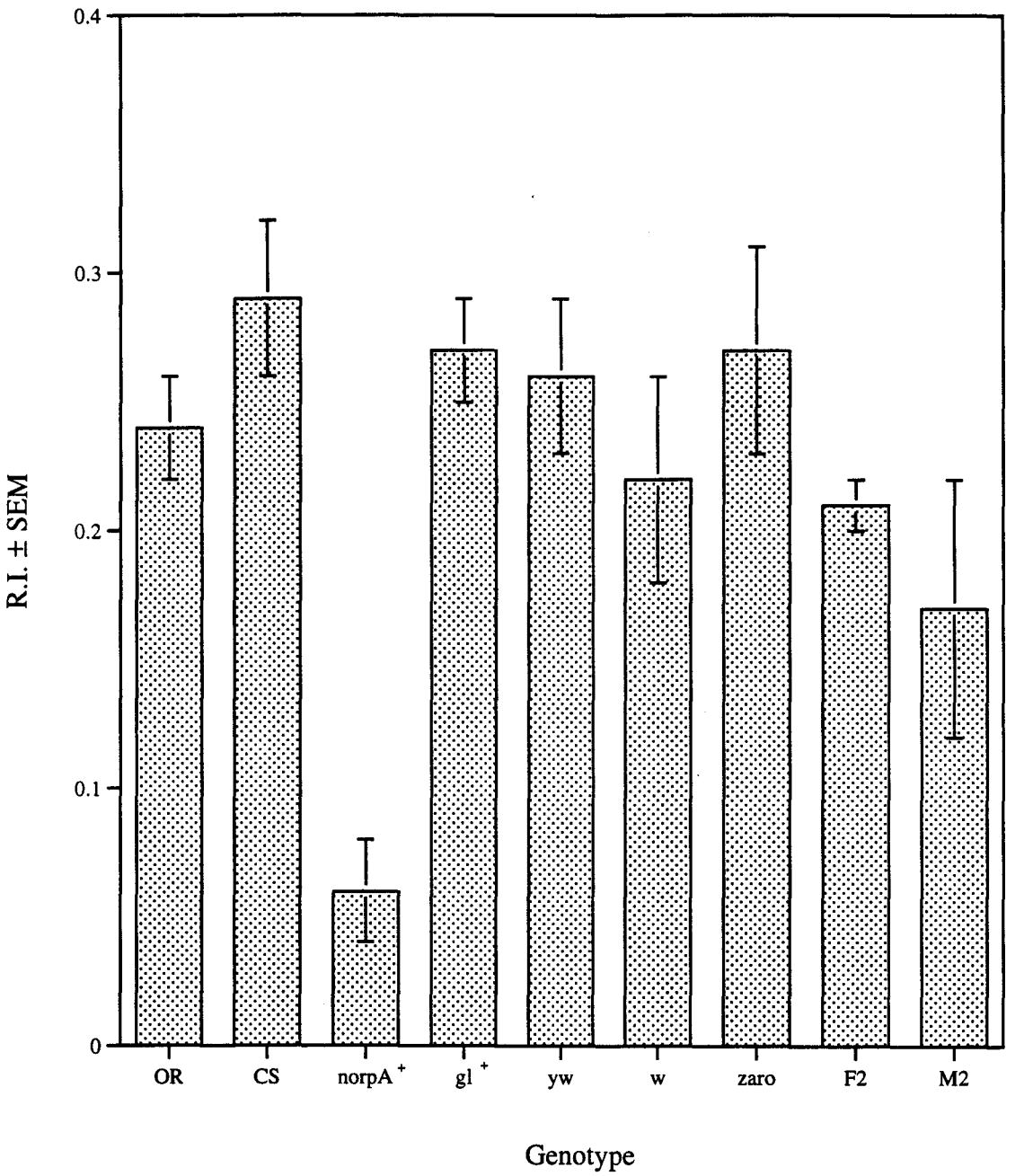
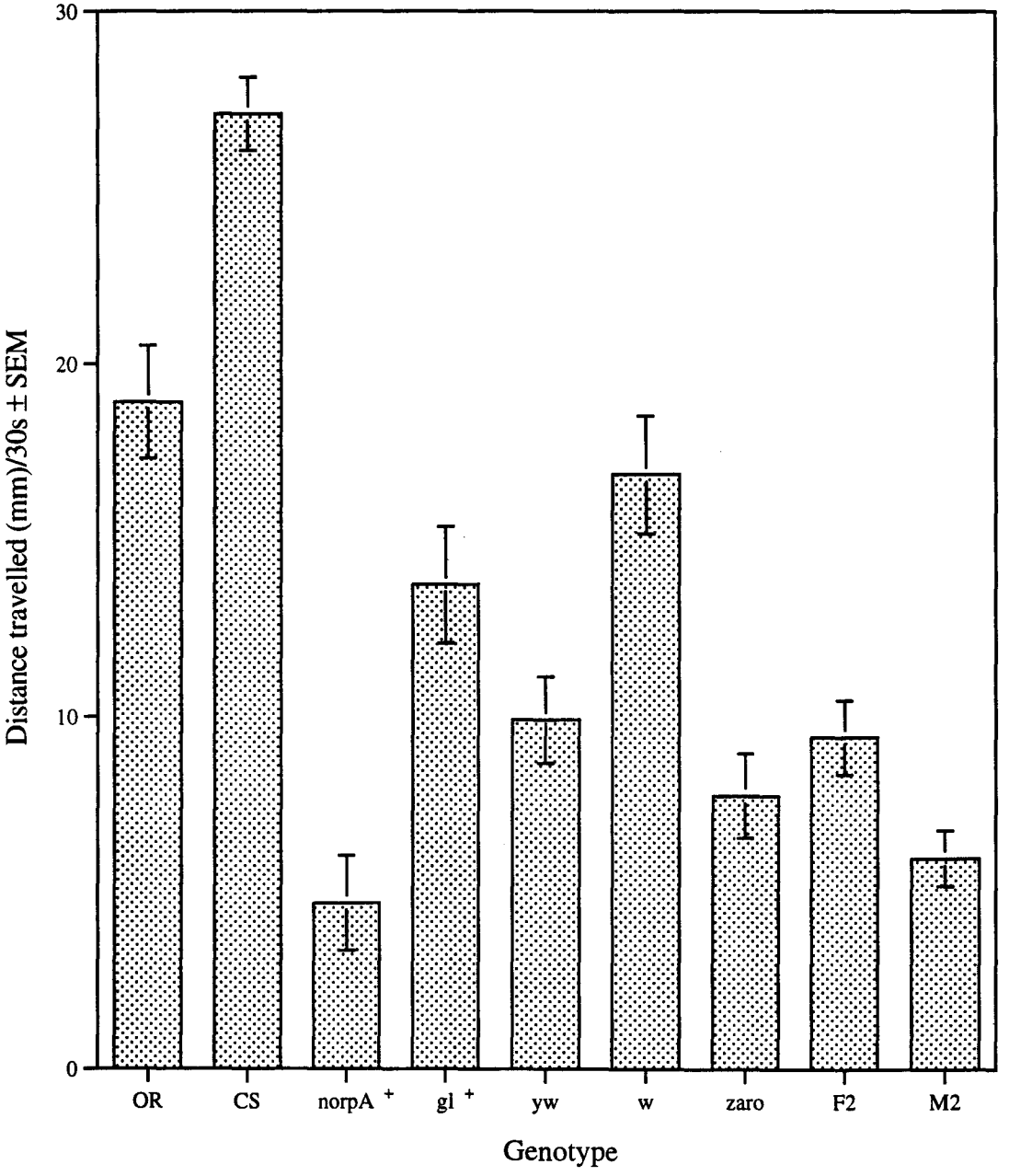

0.4
0.3
0.2
0.1
0
R.I. ± SEM
OR
CS
norpA +
gl +
yw
w
zaro
F2
M2
Genotype

Figure 21. Distance travelled in 30 seconds by wild type and larvae with P element insertions

Distance travelled in 30 seconds was measured in the ON/OFF assay in strains containing P element insertions (*norpA*$^{+}$-n=15, *gl*$^{+}$-n=16, *zaro*-n=20, M2-n=20, F2-n=20) as well as two strains normally used as hosts for P element mediated transformations (*w* (n=20) and *yw* (n=20)). The strains tested exhibit decreased locomotion when compared to wild type. Only *w* larvae exhibit locomotion indistinguishable from wild type (*OR*-n=30, *CS*-n=30).


30
20
10
0
Distance travelled (mm)/30s ± SEM
OR
CS
norpA +
gl +
yw
w
zaro
F2
M2
Genotype

wild type, other behavioural phenotypes, mainly male-male courtship, have been associated with ectopic expression of the *w* gene (Zhang and Odenwald, 1995; Hing and Carlson, 1996).

Unfortunately, M2 and F2 P element vectors had been transformed into recipient stocks of a *yw* mutant genotype, unlike the *zaro*, gl^{+} and $norpA^{+}$ vectors, which had been injected into *w* mutant stocks. The *y* gene encodes a cuticle pigment and adult locomotory phenotypes have been previously associated with mutations in *y* (Burnet and Connolly, 1974). In fact, it would appear that mutation in *y* also disrupt larval locomotion (Figure 21). Thus, it can not be ascertained with certainty from the above experiments, whether the decrease in locomotion of M2 and F2, is due to the presence of a non functional *y* gene or to insertion of the P element. Conversely, although mini-*w* containing P elements, promote decreased locomotion in the lines tested, it remains unclear whether ectopic expression of *w* is responsible for this phenotype, or whether insertion of exogenous DNA is responsible. Study of a P element not containing the mini-*w* marker in a *w* mutant background would probably distinguish between these possibilities.

Decreased locomotion does not restrict the larva's ability to respond to light

One fact that became clear during the search for causes of the apparently P element induced decrease in locomotion, is that these larvae were still testable in the ON/OFF assay. Although locomotion is an essential component of the larva's response to light, as measured in these assays, larvae with decreased locomotory scores can still

display a photoresponse. All the P element containing lines tested, have reduced locomotion when compared to wild type (Figure 21). However, with the exception of *norpA*$^{+}$, these strains all have R.I.'s in the ON/OFF assay which are not different from wild type (ANOVA $F_{(8,183)}$= 10.44, $p<0.001$) (Figure 20). This does not imply that a larva with normal locomotion will necessarily have a wild type R.I.. *norpA*^{P24} and *ninaC* mutant larvae travel approximately the same distance as wild type in 30 seconds. However, they exhibit R.I.'s which are significantly lower than wild type (Figure 5). Thus, although a minimum locomotory threshold must be surpassed before the larval visual response, as measured in the ON/OFF assay, can be quantified, it need not be indistinguishable from wild type. Indeed because the ON/OFF assay makes internal comparisons in length of path travelled, head swings and change of direction, fluctuations in locomotion are compensated. The Checker assay on the other hand is more sensitive to locomotory deficits as the larva has to travel set distances (1cm) between checks.

Chapter V

Discussion

Since the inception of the field of *Drosophila* neurogenetics in the late 1960's with the work of people like Seymour Benzer and William Pak, research in the area has grown tremendously (Benzer, 1967; Pak *et al*, 1969). In particular, the use of behavioural assays in genetic screens, has met with great success in isolating mutations that disrupt assembly and function of the nervous system. In this manner, mutations which disrupt olfaction (Fuyama, 1976; Rodrigues and Siddiqi, 1978; Aceves-Pina and Quinn, 1979; Siddiqi, 1987; Helfand and Carlson, 1989; Woodard et al, 1989; Lilly and Carlson, 1990; Anholt et al, 1996), audition (Eberl *et al*, 1997), grooming (Phillis *et al*, 1993), and circadian rhythms (Konopka and Benzer, 1971; Dushay et al, 1990; Konopka et al, 1991) among others have been identified.

One of the more widely studied behaviours in *Drosophila* is visual response, chiefly because of the ease of scoring and the viability of flies with visual system defects. The use of behavioural paradigms has led to the isolation and identification of a number of genes involved in adult phototransduction, as well as visual system assembly and maintenance (Benzer, 1967; Pak *et al*, 1969; system Harris *et al*, 1976; Hotta and Benzer, 1970; Pak et al, 1970; Gordesky-Gold et al, 1995).

However, much of this work has been done in adults and the correlation between gene function at the adult and larval stages has not been well characterized.

Drosophila is a holometabolous insect where many larval structures degenerate and adult structures are assembled anew. However, this is not true in the nervous system where the adult nervous system forms around a scaffold of larval sensory neurons, motoneurons and interneurons (reviewed in Levine *et al*, 1995). As a result, the genes that affect vision can be hypothetically grouped as: (a) genes that affect and function solely in the adult visual system, (b) genes involved solely in the larval visual system and (c) genes that are common to both the adult and larval visual systems. For example, while at least three of the six known opsin genes are expressed in the larval Bolwig's organ as well as in the adult photoreceptors, a minimum of one is expressed only in adult photoreceptors (Pollock and Benzer, 1988). Mutations in genes involved primarily in visual system assembly, such as *gl*, are expected to have detrimental effects in both larval and adult systems (Moses *et al*, 1989).

In order to initiate a genetic dissection of the larval response to light two new single larval assays were designed: the Checker and ON/OFF assays. Unlike previously used population assays, these assays permit the study of discrete behaviours in a single larva. Additionally, these two assays are distinct in the demands they place upon the larva and yet, complementary in the information that can be obtained from each.

As in all cases where new assays are being employed, the initial work centred on optimizing the assays and resolving any real or perceived shortcomings inherent to them. Initial experiments demonstrated that while larvae do not perceive light above 610 nm

response to wavelengths, less than 600 nm is generally robust. However, two concerns remained: expanding the test window of the assay and the effect decreased locomotion would have on the ability to quantify visual behaviours as measured by these assays.

The *Drosophila* larva's 96 hour life span is divided into several discrete stages: three larval instars separated by two molts (Demerec and Kaufman, 1940). At 25°C, the optimal temperature for *Drosophila* development, the first larval instar commences at hatching, approximately 24 hours after egg lay (AEL). First instar lasts 24 hours (24 - 48 hours AEL) and is separated from the second 24 hour instar (48 - 72 hours AEL) by a larval molt. Third instar, also separated from second by a larval molt, lasts approximately 48 hours (72 - 120 hours AEL). During this time the larva displays a marked aversion to light until approximately late in third instar, when its behaviour becomes photopositive (Grossfield, 1978; Godoy-Herrera *et al*, 1992. This change from photonegative to photopositive behaviour however has not been confirmed in the lab (Sawin-McCormack *et al*, 1995).

Testing in the Checker and ON/OFF assays, was initially performed during third instar at 84 - 86 hours AEL as described in Sawin-McCormack *et al.* (1995). It soon became apparent that this narrow testing window would be too constricting if a genetic screen was to be performed. As a result, a set of experiments were done which tested larval response to light in the ON/OFF assay at six hour intervals from 80 - 98 hours AEL. Testing was staggered such that, two of the time points took place during lights on of a 12 hour light-dark (L/D), and two during light off. This experiment was repeated twice

more, once in a 12 hour L/D cycle opposite ("reverse") to that normally employed ("regular") and once in complete darkness ("freerun").

The results of these experiments showed that larval visual response, as measured in the ON/OFF assay, did not vary significantly throughout the 80-98 hour AEL period in either the "regular" or "reverse" cycles. In addition, locomotion, as measured by distance travelled in 30 seconds, remained fairly constant at each of the time points examined. Larvae raised in complete darkness, however, exhibit response indices (R.I.'s) at 80 and 86 hours AEL which were significantly lower than that exhibited at any other time point tested. While this result would seem to indicate a certain rhythmicity to R.I., possibly masked in the presence of L/D cycles, this remains inconclusive. Further testing over a period of two or more L/D cycles would be needed before any conclusion could be drawn. However, for the purposes of testing in these assays, these results indicate that visual response can be tested in third instar larvae at any time between 80 - 98 hours, so long as larvae are raised in 12 hour L/D cycles.

The Checker and ON/OFF assays although both testing larval visual response, do so in fundamentally different ways. The Checker assay tests the larva's preference for a dark environment. Here, the larva is simultaneously presented with two environments: one dark, one light. It is up to the larva whether it spends the greater proportion of its time in the light or dark squares. In the ON/OFF assay, the larva is presented with subsequent pulses of light and dark. Thus, this assay estimates the modulation of larval behaviour, in this case pattern of locomotion, in the presence of light.

Although essentially different, both these assays demand that the larvae being tested exhibit some degree of locomotion. Larvae that do not move can not be tested. The features of the Checker assay make this locomotory requirement more rigorous than that in the ON/OFF assay. In the Checker assay, the larva responds to light by modulating its locomotion such that it remains in its preferred dark environment. The larva is seen to approach the dark-light boundary and in most instances, perform a series of maneuvers such that it can either return to or remain in the dark environment. As a result, mutations which disrupt locomotion could lead to isolation of false negatives. If the larva does not move, it never leaves the centre dark check and yields a R.I. of 1, indicating that the larva is very photonegative. This may or may not be true.

In order to distinguish between these possibilities, two indices are derived in the Checker assay. The first of these, $R.I.^{off}$, is derived from residence time in light and dark checks in the absence of light. Its counterpart, $R.I.^{on}$, is measured when the light is turned on. Larvae that are able to respond to light by performing the complex exploratory and turning behaviours required, display a $R.I.^{on}$ which is significantly greater than $R.I.^{off}$.

The ON/OFF assay although more lax in its locomotory requirement, still demands that a minimum locomotion threshold be surpassed. As R.I. in this assay consists of an internal comparison of distance travelled in the light and dark by the same larva, decreases in locomotion are automatically compensated. However, in order to gauge the 'wellness' of a larva from a locomotory point of view, distance travelled in 30 seconds was measured. The rationale behind this measurement being that larvae which are 'sick' (i.e. have some sort of locomotory deficit) will perform poorly. Reduced locomotion however,

does not necessarily correlate with a reduction in R.I. in either the Checker or ON/OFF assays. This indicates that larval visual response, as measured in this assay, does not depend solely on the larva's ability to move. Failure to respond in these assays is most likely due to a specific defect in the visual system and not an effect of genetic background. An additional component that makes these assays useful, is that larvae which exhibit severe locomotory deficits can be quickly set aside to be studied at a later time.

However, locomotion is still the main component of larval visual response as measured in these assays. As a result, it was imperative to define not only locomotion but also the means by which the larva appeared to respond to light in each of these assays. The *Drosophila* larva moves about its environment by a series of peristaltic contractions. The head moves forward very quickly as a result of a wave of extension, running anteriorly from the posterior segments (Sawin *et al*, 1994; Berrigan and Pepin, 1995). The head then proceeds downward and the larva established an anchor by digging its mouth hooks into the substrate. The rest of the body then follows the anchored front end, by a series of peristaltic contractions of the more posterior segments, until the whole body is contracted. The next movement begins by the rapid forward motion of the head and culminates with contraction of the entire larval body. In this manner, the larva continues to move forward with little observed lateral displacement of the body (Berrigan and Pepin, 1995). Each cycle of extension and contraction together define stride length.

In the absence of stimulus, the larva has a tendency to travel in a straight line. However, in the presence of light the largely photonegative larva, modulates its locomotion in an attempt to avoid being in the light. It can accomplish this by either

directly increasing/decreasing speed or turning rate (defined as kinesis), or by orienting its body toward or away from the source of stimulation (defined at taxis) (Fraenkel and Gunn, 1961). In fact, at the level of resolution allowed by these assays, the larva appears to be capable of exhibiting both taxis and kinesis.

In order for the organism to be able to perceive and respond to light, either by performing a taxis or a kinesis, it needs to possess a minimum of one light sensing organ. In *Drosophila,* the larval visual system is comprised of two bilaterally positioned groups of photoreceptor cells located just anterior to the cephalopharyngeal skeleton (Steller *et al,* 1987). As a result, the larva is able to not only to perceive changes in light intensity over time but also to make directional comparisons.

In the Checker assay the larva reacts to light by changing its orientations, such that it is positioned away from the source of stimulation, a behaviour defined as taxis (Fraenkel and Gunn, 1961). The result of this behaviour is seen as increased residence time in dark checks. However, it remains unclear whether the larva is performing the comparisons in light intensity temporally (klinotaxis) or spatially (tropotaxis).

In the ON/OFF assay, the larva reacts to light by directly modulating its rate of locomotion, a behaviour defined as kinesis (Fraenkel and Gunn, 1961). The reduction in path length during the light pulse can be attributed to a number of factors: (a) the larva could modulate its stride, usually by increasing stride frequency not stride amplitude, so as to increase speed (orthokinesis) (Berrigan and Pepin, 1995) and/or (b) it could increase the rate of its exploratory movements, namely head swinging followed by a change of direction (klinokinesis) (Green *et al,* 1983; Fraenkel and Gunn, 1961). At the level of

resolution available for this assay at this time, it is unclear whether the larva reacts to light in a manner consistent with orthokinesis. However, the increase in both head swinging and change of direction during the lights ON pulse display, features consistent with klinokinesis.

In all cases examined, mutations in genes involved in adult phototransduction, attenuated all or part of the larval response to light as measured in these assays. In some cases (*norpA, ninaC*) mutations abolished most quantified responses. Other times only subsets of responses were disrupted by mutations in these genes (*ninaE, trp*).

Mutations in the *norpA* gene, completely abolish the larval response to light in the Checker assay, but only a subset of responses quantified in the ON/OFF assay. The *norpA* gene, encodes a phospholipase C, whose expression is essential in but not limited to, the adult compound eye (Bloomquist *et al*, 1988; McKay *et al*, 1995). In the eye, the *norpA* gene product (generated from transcript subtype I) functions as a component in the G protein mediated phototransduction cascade (reviewed by Ranganathan *et al*, 1995; Zhu *et al*, 1994). A second transcript (subtype II) generated by alternative splicing and expressed outside the adult retina is thought to function in other signal transduction pathways (Zhu *et al*, 1993; Kim *et al*, 1995). Initial results with a *norpA* rescue strain, *norpA*$^{+}$, failed to rescue the larval visual phenotype measured by these assays. It is unclear whether this is a result of the transcript used (subtype I) or an effect of point of insertion. Further experiments have to be conducted to distinguish between these possibilities. As a result, it remains unclear whether the larval visual response being measured is mediated by one or both of these isoforms.

Mutations in the *ninaC*^{5} gene, like *norpA*, abolish response in the Checker assay and a subset of responses in the ON/OFF assay. *ninaC*^{2}, however, while not tested in the Checker assay, in the ON/OFF assay exhibits responses similar to those of wild type larvae. The *ninaC* gene encodes two photoreceptor cell-specific proteins, each of which is composed of a protein kinase and a myosin head domain, but which differ at their C termini (Montell and Rubin, 1988). The 132 kD protein, (p132) is expressed primarily in the cytoplasm and the 174 kD protein (p174) is expressed predominantly in the rhabdomeres of adult photoreceptor cells (Porter *et al*, 1992; Hicks and Williams, 1992). While *ninaC*^{5} has reduced levels of both isoforms, *ninaC*^{2} has low levels only of p174. Therefore, the wild type response seen in *ninaC*^{2} mutant larvae but not *ninaC*^{5} larvae indicates that p132, not p174, is required for the larval response to light as measured by R.I. and head swings. The only measure in which *ninaC*^{2} does not respond like wild type is when quantifying change of direction. The larvae are able to perceive and respond to light transitions however, neither of these (either D→L or L→D) is significantly different from change of direction in the absence of transitions(D→D). This result would seem to indicate that in this instance there is a requirement for p174. While Montell and Rubin (1988) did not detect any *ninaC* expression prior to early pupa (day 8), results obtained in this study indicate a larval requirement for *ninaC* encoded proteins. An additional experiment with larvae possessing normal levels of p174 but reduced levels of p132, could confirm this larval requirement.

Mutations in the *ninaE* gene, unlike those described in the *norpA* and *ninaC* genes, do not seem to adversely affect the larval response to light. The *ninaE* gene encodes the

major *Drosophila* opsin, Rh1 and in adults, it is expressed in the outer photoreceptors, R1 - R6 (O'Tousa *et al*, 1985). These photoreceptors are believed to be responsible for image formation while the inner photoreceptors, R7 and R8, may represent a high acuity system (reviewed in Hardie, 1985).

It is known that in *Drosophila* different rhodopsins mediate different aspects of the adult response to light. The R7 and R8 photoreceptor cells expressing Rh3/Rh4 and Rh5/Rh6 opsins respectively mediate some aspects of slow phototaxis and fast phototaxis in high intensity light (Hu and Stark, 1977; Heisenberg and Buchner, 1977; Fischbach, 1979; Hu and Stark, 1980). In dim light however, the Rh1 expressing R1 - R6 photoreceptor cells, mediate fast phototaxis (Heisenberg and Buchner, 1977; Miller *et al*, 1981). In addition, Rh1 also mediates optomotor response, landing and fixation as well as some aspects of slow phototaxis (Heisenberg and Buchner, 1977; Coombe, 1984). It is therefore not unreasonable that different aspects of the larval response to light may likewise be mediated by different opsins. Indeed, the fact that change of direction from L→D is not different from D→D in larvae which carry mutations in the *ninaE* gene (Rh1) seems to suggest a similar mechanism operating in larvae.

R.I.'s, in both the Checker and ON/OFF assays, head swings and change of direction during light transitions are all very similar to wild type. These results indicate that the blue absorbing Rh1 is not necessary for these responses. It is possible that in larvae, the UV sensitive Rh3 and/or Rh4 mediate the responses to light seen. In adults, Rh3 is expressed in approximately one third of R7 photoreceptor cells, while Rh4 is expressed in the remaining two thirds, although it is not known whether this differential

expression pattern exists in larvae (Montell *et al*, 1987; Zuker *et al*, 1987; Fortini and Rubin, 1990). This is unlikely as the light sources used lack the UV component of the spectrum. In addition, it is possible that cells expressing the blue-green absorbing Rh5 and Rh6 opsins, normally expressed in non overlapping sets of R8 cells, could also mediate these responses (Papatsenko *et al*, 1997). However, larval expression patterns of these Rh5 and Rh6 genes have not yet been studied.

One of the last components of the phototransduction cascade is encoded by the *trp* gene. The *trp* locus encodes a protein which is believed to function as a subunit of one or more Ca^{2+} channels (Hardie and Minke, 1992; for review see Friel, 1996). Whether it does so by forming homomeric or heteromeric channels is yet unknown. Briefly, TRP acts as a Ca^{2+} transporter, which refills intracellular calcium stores during light stimulation (Hardie and Minke, 1995). In *trp* mutants adult photoreceptor cells are able to display normal responses to weak stimulation, but are unable to maintain receptor potential in maintained bright stimuli (Hardie and Minke, 1992). Lack of response to light by larvae with mutations in the *trp* gene indicates that larval photoreceptors also have a requirement for the *trp* encoded Ca^{2+} channels. However, although most responses measured in the ON/OFF assay are abolished, *trp* mutants still display increased head swinging in the light pulse, a result for which I have no explanation.

In addition to an increase in head swinging during lights on, larvae which display high R.I.'s also have a propensity to change direction to a greater extent at the boundary from lights off to on (D→L) than from on to off (L→D). This can be interpreted as a temporal comparison of light intensity which is abolished by mutations in the *norpA,*

ninaC and *trp* genes. Larvae with mutation in the Rh1 expressing *ninaE* gene, are still able to make this comparison and modulate their locomotion accordingly. In addition, larvae in which the assembly of Bolwig's organ has been disrupted, do not display this increased change of direction at the D→L boundary. Taken together, these results suggest that many but not all, of the components of the adult phototransduction cascade also possess a function in larvae, and moreover, that Bolwig's organ is necessary for larval visual response as described thus far.

In wild type strains however, change of direction at the L→D boundary was still greater that seen in the absence of light transitions. Two possibilities arose: (a) the change in direction above baseline (D→D) at the L→D transition was due to a startle response, or (b) this response represented a light sensing mechanism independent of the major visual pathway. Support for the latter hypothesis comes from the fact that mutations, such as *norpA, ninaC* and *gl*, which abolish larval photoresponse still display a change of direction in the absence of transitions less that at the L→D transition.

However, this response can not apparently be mediated by rhodopsins other than expressed by the *ninaE* gene, or Ca^{2+} channels not composed of TRP subunits. In addition, results indicate that wherever these extra light sensing cells are housed, they must arise as a consequence of early expression of the *so* gene. The *so* gene encodes a homeodomain protein which has been shown to be required for visual system development (Cheyette *et al*, 1994; Serikaku and O'Tousa, 1994).

Together, these results suggest that although this basic visual system is not housed within the *gl* expressing cells defined as photoreceptors, the cells that do house it possibly arose from common progenitor cells, and as a result are probably located very close to the traditional photoreceptors. Although these results do not exactly coincide with what is known about the expression pattern of *ninaE*, it is possible that *ninaE* is expressed at low levels outside the Bolwig's organ and in a *gl* independent manner. In addition to the previously described photic input pathway in flies which does not rely on adult phototransduction for the entrainment of circadian rhythms (Wheeler *et al*, 1993) extra retinal perception has been described in many vertebrates and invertebrates (Oliver and Bayle, 1982; Page, 1982; Underwood and Groos, 1982; Garcia-Fernandez *et al*, 1997).

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