Characterization of the Blackpatch mutation in Drosophila melanogaster

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Characterization of the *Blackpatch* mutation in *Drosophila melanogaster*

by

Mythreyi D. Shastri

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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This is to certify that the Doctoral dissertation of

Mythreyi D. Shastri

has met the dissertation requirements of Iowa State University

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Major Professor

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For the Major Program

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For the Graduate College
DEDICATION

This thesis is dedicated to my parents in grateful thanks
for their constant support and encouragement.
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CHAPTER 1. GENERAL INTRODUCTION

In 1899 E. B. Wilson identified four basic questions of development as, a) cell determination, b) cell differentiation c) pattern formation, and d) localization of developmental potential in the egg. At that time it was recognized that these four represented the key questions that needed to be answered to understand cell behavior during development. Wilson understood from detailed studies of embryonic development how cells behaved during development, but what he did not understand was how cell behavior was regulated. Today we understand that genes are responsible for regulating development. The role of genes in controlling development was established early in the twentieth century by investigators who observed that mutant alleles of genes could alter fundamental developmental processes. W. E. Castle and C. C. Little in 1910 (Wilkins, 1986), identified that the yellow gene in mice gives a dominant coat color phenotype. Recessive homozygous alleles also give an embryonic lethal phenotype. Understanding of this latter aspect showed that certain genes have a function during development.

The question of genetic control of development was further refined by T.H. Morgan, who in 1936 proposed that the developmental state of a cell is controlled at any given time by the activity of a particular set of regulatory genes. Interactions of the cell with other cells or the environment cause changes in the set of active genes. Some active genes are repressed and some repressed genes are activated, generating a new set of active genes and a new developmental state for the cell. Morgan proposed that a series or progression of such changes in gene regulation could control the changes in the developmental state of cells that occur throughout the development of the organism. The fundamental questions of Wilson thus became questions of, which sets of genes control which cells at each stage of development, and how are the activities of these genes.
regulated. The answer to these questions can be found by identifying individual genes that regulate particular developmental events or processes and by investigating their function.

An ambitious and direct approach at identifying "all" of the genes that regulate one stage of development was taken by C. Nusslein-Volhaard and E. Weischaus (1980). They performed a saturation mutagenesis screen for mutations in genes regulating segmentation in *Drosophila*. Their results identified a series of segmentation genes, and established our modern concept of developmental regulation by gene hierarchies. During development, a sequential action of regulatory genes gradually restricts the developmental potential of pluripotential cells, assigning them determined states. The genes involved in determination and differentiation show a spatial and temporal specificity of action that reflects their specific role in the process. The role of each gene can be understood by studying the changes in development caused by mutant alleles of that gene.

Another fundamental principle of developmental genetics is that genes that can mutate to give a lethal mutant phenotype represent genes with important functions. While there are many genes with vital "housekeeping" functions, developmental regulatory genes with essential functions can be identified by isolating mutations that kill cells or organisms at particular developmental stages. The normal role of the wild type alleles of these genes can be identified by studying the details of the lethal phenotype. The study of lethal mutant alleles often involves three steps. First, the time of death, called the lethal period or the lethal phase, of mutant individuals is identified. Second, the differences between the normal and the mutant individuals for the various organs and tissues at the time of death are described. Third, the earliest time of appearance of abnormalities in these tissues or organs is noted. The general principle is that the systems showing the first evidence of abnormality are those in which the normal function of the genes is needed. In *Drosophila* these types of analyses are aided by the application of genetic analytical techniques, such as
induced somatic mosaics and temperature-sensitive alleles, that can also help determine the tissue, organ, or cell type in which a gene acts.

*Blackpatch* is an example of a gene whose normal function is essential for development. Mutations of *Blackpatch* can cause the death of the organism during the larval period when homozygous, and can cause the death of specific cells in the adult visual system when heterozygous. The fact that there are (at least) two different periods during which *Blackpatch* functions, and that there are different effects in homozygotes and heterozygotes is not unusual. Genes frequently exhibit pleiotropy and are required in different times and for different aspects of development. In order to understand the nature of the function of *Blackpatch* it is essential that the various aspects of the phenotypes given by mutant alleles be well characterized. This is the main goal of the experiments reported in this thesis.

**Dissertation Organization**

The main theme of this dissertation is the characterization of the effects of *Blackpatch* mutations on the adult visual system and on larval development. The thesis is divided into six chapters. Chapter I is the general introduction. Chapter II is the characterization of the *Bpt* mutation in the visual system. Chapter III gives information about the characterization of the recessive lethality. Chapter IV talks about the two approaches used to clone the *Bpt* gene. Chapters II, III and IV are written in the format of a manuscript to the journal, *Genetica*. Chapter V is a paper titled, "Prolonged exposure to carbon dioxide does not have long term effects on the electroretinograms in Drosophila". This paper represents a technical advance that was developed during the course of this work. It is published in *Drosophila* Information Service 80. Chapter VI is the general conclusions.
Background and Significance

Structure and Development of the Visual System

The major part of the thesis deals with analyses of the effects of Blackpatch mutations on the developing adult visual system. Before discussing these effects it will be helpful to review the normal structure and development of this system. The adult visual system of Drosophila is a complex retinotopic array conveying information about the outside world in a precise spatial manner (reviewed by Kunes and Steller, 1993). It consists of 2 compound eyes that have a retina overlying 3 optic lobes of the brain. The retina (also referred to as the ‘eye’) is a complex tissue with several types of cells including photoreceptor cells, pigment cells and cone cells. Immediately underneath the retina is the first optic lobe, the lamina followed by the medulla, lobula and the lobula plate.

Structure of the retina

The retina in Drosophila has come to the serious attention of developmental geneticists and in recent years, the information regarding its development and pattern formation has exploded. The retina of each compound eye consists of 750 to 800 ‘simple eyes’ or ommatidia (reviewed by Wolff and Ready, 1993). Each ommatidium consists of 8 photoreceptor cells called R1 through R8. Six of these, R1 through R6 are arranged in an hexagon so that their inner light sensitive surfaces, called rhabdomeres, form a column. R7 and R8 are found inside this hexagonal column with the R7 photoreceptor stacked on top of the R8. Hence they are called the ‘inner photoreceptors’. On the external surface each ommatidium has a corneal lens underneath which is the pseudocone. The pseudocone consists of a refractile gel. Four cone cells are present under the pseudocone. The ommatidium also contains 2 primary pigment cells flanking the cone cells anteriorly and posteriorly. Around this six secondary pigment cells form a hexagon. In the 6 vertices, 3
tertiary pigment cells and 3 bristle cells alternate with each other. These are the components of every ommatidium and together they form a perfect lattice-like structure.

**Development of the retina**

The formation of the adult eye begins in the eye-antennal imaginal disc during the third larval instar (reviewed by Wolff and Ready, 1993). The eye-antennal disc begins as an invagination of the embryonic ectoderm. The eye disc is at first an unpatterned monolayer of cells. During larval development, the disc cells divide by mitosis and the disc increases in size. During the third instar, there appears a groove in the eye disc, called the morphogenetic furrow. This begins in the posterior margin and travels anteriorly, like a wave. At the posterior edge of the morphogenetic furrow, sequential recruitment of cells leads to ommatidia formation. The earliest pattern of cells is seen at this edge in the form of a rosette of 5 cells. It is not clear how this rosette becomes the 5 cell precluster, the 5 cells that will later become the photoreceptor cells. The cells develop their identity by receiving and interpreting positional information (Ready, Hanson, and Benzer, 1976; Ready, 1989; Cagan and Ready, 1989; Banerjee and Zipursky, 1990; Cagan, 1993). The R8 cells express their neural identity first (Tomlinson and Ready, 1987). This is followed by R2/R5, then by the R3/R4 pair and lastly by the R1/R6 pair. The cell that is in contact with all these photoreceptor cells differentiates into R7. By the end of the third larval instar most of the ommatidia have developed. A few more are added in the young pupa.

The remaining non-neural cells that function to maintain the lattice-like structure of the compound eye are added in the pupal stages of development. At first the anterior and posterior cone cells meet above the photoreceptor cells. This contact continues downwards so that the R1, R6 and R7 cells form one group and the R2, R3, and R4 form another group. Twenty hours later, the equatorial and polar cone cells develop such that, they meet on top of the photoreceptor cells separating the anterior and posterior cone cells. The anterior and posterior cone cells continue to contact each other at the base of the
ommatidium. At 22 hours, the 2 primary pigment cells begin to develop. They are seen behind the anterior and posterior cone cells. They continue to develop more basally and run along the entire length of the anterior and posterior cone cells. In the apical region, the 2 primary pigment cells start to grow outward. They meet at about the middle of the equatorial and polar cone cells and so, encompass all the 4 cone cells. They form the primary pigment cell collar. The nuclei rise to the top. By 48 hours in pupariation, the primary pigment cells have completed development. Due to all this cell growth, other cells are pushed into the inter-ommatidial space. Secondary and tertiary pigment cells develop based on their contacts with neighboring cells. Cells in contact with the photoreceptors basally and in contact with the primaries apically become secondary pigment cells. Two secondary pigment cells are formed initially and later, by the end of pupariation, one of them is removed by cell death (Cagan and Ready, 1989; Wolff and Ready, 1991). A cell in contact with 3 primary pigment cells of 3 different ommatidia becomes the tertiary pigment cell. Most probably, 3 potential tertiaries are found at the vertices and 2 of these die leaving one tertiary pigment cell. In the pupa, between 60 and 160 hours of development, each of these cells become further specialized.

Genes controlling development of the retina

Several genes are involved in every aspect of eye development. A review by Yamamoto (1993) lists 20 genes involved in photoreceptor cell fate determination alone. Events during ommatidia formation: the correct number of cells adopting a neural cell fate, establishment of correct cell identities and the correct spacing of the ommatidia behind the furrow, all require the function of specific genes. These genes have varied functions. For example, yan functions as a negative regulator to prevent photoreceptor cell fate determination (Lai and Rubin, 1992).

The first cell that differentiates, the R8 cell, expresses boss (Hafen, et al., 1993). The boss gene product is required for recruiting R7. R8 also needs to express rap that is
necessary for the regular initiation of ommatidia in the furrow. R2 and R5 are recruited and they express rough. rough is required for the identity of R2 and R5. R1 and R6 are recruited and begin to express Star. R7 expresses sevenless. This is a cell surface receptor whose ligand is boss. The dialog between sevenless and boss is necessary for R7 to assume its identity. In sina mutants, the R7 precursor cell becomes a non neuronal cone cell indicating that R7 requires expression of sina. Genes like roughest are needed for the programmed cell death of additional secondary pigment cells. The involvement of DER, Drk, GAP1, raf, Ras1, sev, and Sos is especially interesting because this set of genes works in a signal transduction pathway to specify the terminal structures in the developing embryos, for eye development and ventral cell fate determination (Hafen and Dickson, 1993). It appears as if the entire sequence is used in all these different pathways. However further and finer dissection of the different functional components of each of these genes will reveal the real similarities and differences between these pathways.

The components of the signal transduction pathway are as follows: The boss protein, a membrane bound transmembrane protein, is expressed in R8. It serves as a ligand for the sev protein, a receptor tyrosine kinase expressed in several cells including R7. The action of sev is to turn on Ras1, a GDP binding protein through Drk, Sos and Gap1. Activation of Ras1 activates raf, a serine-threonine kinase that in turn activates rolled. This protein acts on another downstream gene, sina whose protein is localized to the nucleus. It is suggested that sina may be the target and its function is necessary for the determination of the R7 cells. The sevenless pathway suggests three mechanisms of control exerted by genes in pattern formation. Ultimately, all 3 are probably necessary for normal development. The 3 mechanisms are, a) restricted expression of the ligand and receptor, b) acquisition of the competence to respond to the signal, and c) the presence of both positive and negative inducing signals that fine tune the whole process.
The appreciation of the events leading to the determination of one cell in the retina is a harbinger of similar processes in optic lobe formation. Given the complexity of the structure of the optic lobe and the precise retinotopic interaction of the tissues that arise independently to form the visual system, it will not be surprising to find similar or more complex signal transduction pathways involved.

*Structure of the lamina*

Some of the earliest work in describing the structure of the insect visual system was accomplished in the turn of the century by Vigier, Ramon y Cajal and Sanchez. From the very beginning, the idea of understanding the complex structure as a tool to understand the function was recognized. They had already identified the structure of the photoreceptor cells and its synapses with the second order neurons. Cajal used the Golgi staining technique and described this region of synapse as the intermediate retina and in this region, the synapses occur in the optical cartridges. Cajal had identified that the photoreceptor axons synapse with contiguous cartridges. O. Trujillo-Cenoz (1965), studied the intermediate retina (what is now called the lamina) of *Sarcophaga bullata* and *Lucilia* using electron microscopy and serial section reconstruction. He was able to describe the two central fibres in the optical cartridge (now called L1 and L2) making extensive synaptic contacts with the 6 photoreceptor axons. Braitenberg (1967) in attempting to explain the optomotor behavior of the fly, *Musca domestica* wanted to bring together anatomical, behavioral and electrophysiological understanding of the insect visual system. He used light microscopy to obtain a broader field of vision and confirmed Trujillo-Cenoz's findings. Instead of two central fibres in the lamina, Braitenberg describes 3 fibres, L1, L2 and L3. L1 and L2 arise from cell bodies that lie above the lamina. He has shown the entry of the 6 photoreceptor axons into 6 laminar optical cartridges. Each cartridge in turn receives axons from 6 overlying ommatida. The location of the 6 cartridges provides an asymmetrical pattern that corresponds to the set of 6 photoreceptor cells overlying it.
Horridge and Meinertzhagen (1970), using the visual system of *Calliphora vomitoria*, have described the connectivity of the retina-lamina projections in detail by analyzing over 200 photomicrographs to trace 800 axons simultaneously in one region. They were able to also describe errors in the connectivity. Bruce Boschek (1971) described the ultrastructure of the retina and lamina for *Musca domestica* by examining cross-sections through the tissues and observing the morphology of the cells. N. J. Strausfeld (1971) did an extensive light microscopy study on the visual system of five other dipteran insects, *Calliphora erythrocephala, Calliphora phaenicia, Calliphora vomitoria, Musca domestica, Syrphus elegans*, and *Eristalis tenax*. The *Drosophila* lamina has a structure, similar to that described above (Meinertzhagen and O’Neil, 1991, Meinertzhagen and Hanson, 1993). The laminar cartridges consist of the L cells, L1 to L5, that end their axons in the medulla. Some of the other cells are the T1 cells, amacrine cells and glial cells.

**Development of the lamina**

In the embryo, the photosensitive Bolwig’s organ is seen at about 9 hours of development (Meinertzhagen and Hanson, 1993). Bolwig’s organ is connected to the larval brain by Bolwig’s nerve. The later innervation of the optic lobe by axons from the photoreceptors is dependent on this embryonic connection. Photoreceptor cells that develop from the larval eye disc send axons through the optic stalk and innervate optic lobe precursor cells. The development and position of the optic stalk is dependent on the correct projection of the Bolwig’s nerve. The Bolwig’s nerve is a component of the larval visual system originating from a cluster of photoreceptor cells in the third instar larva. It travels through the antennal disc, the eye disc, through the optic stalk and innervates a subset of optic lobe precursor cells in the medial margin of the medulla cortex. Between 24 hours of midthird instar larval life and 24 hours after pupariation, the photoreceptors send out axons.
The photoreceptors in the retina elaborate their axons through the optic stalk and innervate the underlying lamina precursor cells. Elongation of photoreceptor axons takes place in four stages. Initial outgrowth of photoreceptor axons to the lamina starts with the posterior photoreceptor cells and progresses to the anterior photoreceptor cells. This process starts midway in third instar larva and continues until 24 hours post pupariation. The second phase is the growth cone interactions among the photoreceptor axons of the R1 to R6 cells. The interactions commence during very late third instar larva and continue until 22 or 24 hours post pupariation. The next phase of development is, the photoreceptor axons reach the laminar targets between 24 to 48 hours post pupariation. The laminar terminal completes development around 48 hours post pupariation. This completion involves the final patterning of the cartridges with the correct numbers of cells in their right locations. Synaptogenesis in the lamina takes place in the second half of pupal development, i.e. between 48 hours to 96 hours.

Questions regarding the development of the lamina have been addressed by the study of mutations affecting optic lobe development. Invariably, many of the mutations also affect eye development. One of the first questions addressed was whether optic lobe development depends upon this innervation or does it develop autonomously from the retina. Meyerowitz and Kankel (1978) showed that eye development proceeds independently while development of the optic lobe is dependent on the retina. When retinal innervation is prevented, there is massive degeneration of the optic lobe. Four classes of mutations were identified, a) mutations that do not show the normal hexagonal organization of ommatidia, b) mutations with abnormal organization of the axons in the lamina and medulla, c) mutations that disorganize the facet array and also have laminar fibre abnormalities, and d) mutations that reduce or eliminate the eyes altogether. Mutations like Glued, rough, and glass do not have the perfect hexagonal organization of the ommatidia and show abnormalities in the laminar fibre array. Mutations in lozenge show
disorganization of the facets accompanied by abnormalities in laminar fibers (Batterham, 1996). When the eyes are very small or absent the optic lobe is also very small or completely degenerate like in small optic lobes (sol) (Fischbach, 1983; Fischbach and Technau, 1984). A different study on glass mutants has shown that those regions of the optic lobe that receive innervation develop further while other areas do not (Selleck and Steller, 1991). In the sine oculis (so) and Ellipse mutants, normal retinal axons project though neighboring axons are absent (Kunes, Wilson, and Steller, 1993). In so mutants, the medulla and lobula that are not innervated by axons show a great increase in degeneration in the first half of pupal life especially between 30 to 50% of pupal life. There is no excess degeneration when there is normal innervation. Studies on the hedgehog mutation have shown that the secreted protein, hedgehog, is required early to induce neurogenesis in the lamina (Huang and Kunes, 1996).

Experiments with one mutation, disco have shown that signals from the optic lobe are required to maintain the structure of the photoreceptor cells (Campos, Fischbach, and Steller, 1992). The disco mutation has two phenotypes that can be described as the "unconnected" and "connected" phenotype (Steller, Fischbach, and Rubin, 1987). In the unconnected phenotype, the adults have a very rudimentary optic lobe that was not innervated by the photoreceptor axons. The connected phenotype has an optic lobe similar to the size of the wild type optic lobe. However in both phenotypes, the optic lobes are highly disorganized and very different from wild type. Most of the disco mutants have defects in the compound eye as well. Older flies show degeneration of photoreceptor cells that manifest as black spots. All the flies with the unconnected phenotype were blind and had electroretinograms that did not have laminar activity. The defect in the disco mutants lies in the absence of the larval Bolwig's nerve. The adult eye and optic lobe develop as different tissues and establish connections in the third larval instar. In disco mutants, the nerve is ectopic and present outside the eye disc. It is also misrouted and shows no
consistent projection pattern. In the mutants, the Bolwig's nerve never innervates its targets in the optic lobe anlagen. The presence of the optic stalk is dependent on the location of the Bolwig's nerve. Therefore, in disco mutants, the optic stalk is displaced or absent. Sometimes the photoreceptor axons are able to make connections with the optic lobe precursor cells even through a displaced optic stalk. In these, the degeneration is not extensive and the connected phenotype is seen. In the flies where no optic stalk developed, the optic lobe precursors received no innervation and showed extensive degeneration in the pupal stage. This gives the unconnected phenotype. The photoreceptor axons end in a mass of non-neural tissue, hemolymph or muscle or unrecognizable tissue.

Mosaic analysis has shown that the genotype of the compound eye was not important for determining the disco phenotype. Is disco necessary in the eye for continued survival, or is innervation with target cells necessary for continued survival of photoreceptor cells? Mosaic analysis shows that photoreceptor cells, mutant for disco survive without degeneration if they innervate the optic lobe. In flies exhibiting the connected phenotype, the photoreceptor cells did not degenerate while cells with the unconnected phenotype showed degeneration. Electretinograms from flies with the connected phenotype showed laminar activity comparable to wild type. This indicates that although the lamina of connected disco mutants is disorganized, the innervation between the retina and laminar targets is normal and functional. The degeneration in the mutants, however is due to absence of maintenance signals from the optic lobe. This indicates that trophic signals exist between the retina and optic lobe and this type of interaction is required from the target cells for the maintenance of the retinal cells. Mutants of extra eye (ee) also show degeneration in the photoreceptor cells, 15 days after eclosion, specifically in the ectopic eye. The ectopic eye never makes connections with the optic lobe and its axons end in a mass of non-neural tissues. This is in contrast to the normally placed compound eyes that are also mutant for ee, but do not degenerate presumably because their connections to
the optic lobe are normal. Unlike the mutants of the phototransduction pathway, this degeneration is not light dependent. The degeneration is also specific to the photoreceptor cells, since the cone cells and pigment granules of pigment cells are present even after the R cells have degenerated.

**Function of the Visual System**

**Electroretinograms**

Visual transduction is both an electrical and a biochemical process in the photoreceptor cells that converts the light stimulus to electrical impulses. The R1 to R6 are the largest class of photoreceptors in the compound eye and they contain the visual pigment, rhodopsin. Rhodopsin absorbs light at 485 nm and is photoconverted to stable metarhodopsin at 580 nm. This photoexcitation opens up sodium channels causing permeability changes in the photoreceptor membrane that leads to depolarization. The receptor potential is a result of this depolarization (Pak et. al., 1976).

Electroretinograms (ERGs) are representations of the total electrical activity in the retina and lamina. The receptor potential is due to the summation of a large number of quantum bumps, each arising in response to a single photon (Minke, Wu, and Pak, 1975). With increasing stimulus intensities, the average bump rate increases and the bump amplitude decreases when measured at the steady-state phase of receptor potential. The decrease in bump amplitude at steady state with high light stimulus may be responsible for the decay of the receptor potential after its initial peak to the steady state level during light stimulation.

The electroretinogram has “on” and “off” transients, a positive and negative peak in response to the “on” and “off” of the light stimulus. These are the laminar transients. Between the two transients is the corneal negative arising due to the activity of the retinal photoreceptor cells. Heisenberg (1971) was able to functionally isolate the receptor and
laminar potential in the ERG. Coombe (1986) by analyzing the mutant, *Vacuolar medulla* was able to show that the on and off transients arise due to the activity of the L1 and L2 cells in the lamina.

*Mutants (Genes) in phototransduction*

An important tool in assaying mutants of the phototransduction cascade was the electroretinogram (ERG) (Cosens and Manning, 1969; Hotta and Benzer, 1969; Johnson and Pak, 1986). Since the ERG represents the electrical function of the eye, functional mutants in the visual transduction pathway can be isolated by its aberrant effects on the ERG. Several genes identified in this manner have provided information about the biochemical events that follow light stimulation of the *Drosophila* eye. In addition, the cloning and characterization of these genes have provided molecular information about the nature of the proteins in the transduction machinery. Since invertebrates use common signaling pathways for phototransduction as well as for signaling within and between cells, the study of this pathway provides information that is useful in the general terms of signaling mechanisms. Abnormal electroretinograms from visual mutants of *Drosophila* have long been reported (Hotta and Benzer, 1969; Cosens and Manning, 1969; Pak, Grossfield, and Arnold, 1970).

Mutations in the opsin gene, termed *nina A, B,...J* have been identified. The name, *nina* stands for 'neither inactivation nor afterpotential' with regard to its electroretinograms. The *nina* mutants fall into 5 complementation groups termed C, A, B, D, and E. In that order, they show increased reduction in rhodopsin levels as compared to the wild type. In the same order, correlating with the decrease in rhodopsin, there is decrease in the prolonged depolarizing afterpotential (PDA). In wild type flies, the PDA is a prolonged depolarization that persists after the end of the blue light stimulus. The amount of the PDA is quantifiable and hence is a sensitive screen for isolating mutants. In *nina E*, the PDA is absent and the rhodopsin content is 1% of the wild type. Molecular data shows that *nina E*
codes for the opsin portion of the predominant rhodopsin, Rh1. Another mutation in the
visual transduction pathway, norpA is explained in detail later and shows the relationship
between electroretinograms, role in visual transduction and retinal degeneration. In the trp
mutant, the efficiency of quantum bump production is reduced because its amplitude does
not decrease with increasing light intensity. The ERG as a whole decreases to baseline at
high light intensity. The protein is not similar to any known protein and is thought to be
associated with the photoreceptors.

Not all mutants in the pathway affect the phototransduction process. Both nina A
and C affect R1 to 6 rhodopsin and the cytoskeleton of the microvilli that make up the
rhabdomeres. Hotta and Benzer have also described a different set of mutations that cause
retinal degeneration after light exposure. They are the retinal degeneration genes, rdgA,
rdgB, and rdgC. The locus of the mutations in A and B are in the photoreceptor cells. The
ERGs of 7 day old flies show reduced receptor potentials and absence of on transients.
The outer photoreceptors show degeneration 7 days after eclosion, when maintained on 12
hour light-dark cycle (Harris and Stark, 1977). The physiological defect could have
commenced in late pupal life since the photoreceptors are structurally complete at that time.
8 day old rdg C flies also show receptor degeneration and highly reduced ERGs. The rdgC
gene encodes a serine/threonine protein phosphatase that contains multiple calcium binding
sites (Steele, Washburn, Rieger, and O'Tousa, 1992). Several proteins in the eye are
phosphorylated and may use rdgC gene product for regulation. Light stimulus
phosphorylates a protein that becomes toxic if not dephosphorylated later. rdgC gene
product is required to dephosphorylate the protein and in its absence, the eye accumulates a
toxic metabolite and consequently, shows degeneration.

Eye degeneration due to aberrant function

Degeneration is seen in the eyes of mutants that have an aberrant visual transduction
pathway. Such mutants can be isolated by functional assays, for example, by measuring
electroretinograms. These mutants may show degeneration in the retina only or in the lamina. Though the consequence of laminar aberrations have been identified in function, the corresponding laminar degeneration, if any, has not been studied as extensively. This is because the lamina is more difficult to access. Several mutants with defects associated with the phototransduction leading to retinal degeneration have been isolated. Examples are rdgA, rdgB, rdgC, norpA, ninaE, ninaA (Harris and Stark, 1977; Johnson, Frayer, and Stark, 1982; Stark, De-Mao Chen, Johnson and Frayer, 1983; O'Tousa et. al., 1985; Meyertholen, 1987; Bloomquist et. al., 1988; Shieh et. al., 1989). Studies of the norpA mutants and its gene product are a case in point. This mutation had been extensively studied for its electrophysiology and photoreceptor degeneration. At eclosion, norpA mutants do not possess a receptor potential. Using a temperature sensitive allele, norpA<sup>H52</sup>, Wilson and Ostroy (1987) were able to identify that there is continued reduction of the amplitude in the electroretinograms when flies were moved from 17°C to 33°C. The reduction in amplitude was seen only for lower light intensities when the fly was exposed to 33°C for a short interval. But when maintained at 33°C for 26 minutes, the ERG amplitude starts to decrease in response to higher light intensities as well. This decrease in amplitude to light is complete, that is, the ERG is abolished when the fly is maintained at the higher temperature for 50 minutes. The electroretinograms were recovered when flies were moved back to 17°C. These results indicate that the norpA protein is necessary for normal visual transduction.

The norpA mutants also show changes in eye structure. When maintained in the dark, the norpA mutants do not show defects in photoreceptor structure, the deep pseudopupil and rhodopsin concentration. But when the flies were maintained on a 12 hour light - dark cycle, at 24°C, there are several changes. The number of flies having the deep pseudopupil (the reflection of light when it passes through the columnar structures of the retina and lamina; the presence of seven spots of light in a trapezoidal arrangement
argues for the presence of the correct and complete retina) reduces at 6 days of age and by 10 days, the rhodopsin concentration decreases. By 14 days, there is massive degeneration in the eyes with only a few rhabdomeres present in irregularly-arranged ommatidia. In *norpA* mutants, the intra-retinular particles are dispersed instead of showing light induced aggregation (Stark, Sapp, and Carlson, 1989). There are a higher number of “zippers” - striated areas of high membrane density than in the wild type. There is a lower density of P face particles indicating lower concentration of rhodopsin. Unlike the findings of Meyertholen, in this study, they did not find extensive receptor degeneration. These differences may be due to allelic, temperature and light intensity differences. The second study has also shown that the process of turnover of the rhabdomeric membrane are normal in the mutants. Phosphatidylinositol specific phospholipase C was very much reduced in these mutants (Yoshioko, Inoue, and Hotta, 1985). The protein product has extensive similarity to a bovine PI-PLC and so there is strong indication that *norpA* codes for phospholipase C (Bloomquist et al., 1988). It has also been shown that a light dependent G protein-coupled activation of PLC is a part of the phototransduction process (Ranganathan, Harris, and Zuker, 1991; Dolph, et al., 1994). Phospholipase C generates intracellular transmitters and in *norpA* mutants, this reduction may affect the conversion of inositol phosphate (IP) to IP3. Though it is known that a consequence of the phototransduction defect is the disruption of photoreceptors, it is still not clear how any of these mutant effects directly lead to receptor degeneration. Why do mutations in PLC ultimately cause photoreceptor degeneration or why do absence of electroretinograms lead to reduction in rhodopsin concentration? There are no clear answers.

An experiment that shows the relationship between phototransduction and receptor degeneration was conducted by Harris and Stark (1977). Mutants at the *rdgB* locus cause retinal degeneration that is light induced. The R1-6 show reduced integrity and decreasing levels of rhodopsin within a week. The lamina degenerates more quickly than the
photoreceptors. The photoreceptors are functionally defective at eclosion but anatomical changes are seen later. The R7-8 connections in the medulla do not seem to be affected and continue to show electrical activity beyond one week. The retinal degeneration can be blocked by rearing the flies in the dark, depriving them of vitamin A, using genetic means to eliminate acid phosphatase, the lysosomal enzyme, and by constructing double mutants of \textit{rdgB} and \textit{norpA}. The absence of receptor potentials due to the \textit{norpA} mutation prevents retinal degeneration due to \textit{rdgB}.

Retinal degeneration can also be caused by mutations in the opsin gene or by incorrect localization of the opsin gene. In \textit{nina A} mutants, the Rh1 opsin gene is expressed at normal levels but the photoreceptors have only about 10% of wild type levels of rhodopsin. The \textit{nina A} gene codes for a cyclophilin homolog necessary for the conversion of proline containing polypeptides from the cis to the trans form. This activity is necessary for the correct localization of rhodopsin in the photoreceptors. The \textit{nina E} gene codes for rhodopsin and mutants have very small rhabdomeres.

An allele of \textit{nina E} called \textit{ora} has the outer rhabdomeres missing. In this class of mutants, deficiencies in the structure of the photoreceptor cells leads to degeneration. Flies with the \textit{sine oculis} (so) mutation show degeneration of eye imaginal disc cells in the third instar larva. Between 32 and 45% of pupal development, the ganglion cells in the medulla and lobula degenerate. The lamina does not develop in the eyeless condition. In both the mutants, extensive degeneration occurs in the optic lobes in the first half of pupal development. The \textit{small optic lobes} (sol) mutation affects the medulla. Degeneration of the axons in so flies is because of the eyeless condition.

\textbf{Role of Programmed Cell Death in the Development of the Visual System}

Programmed cell death plays an extremely important role in the development of the retina to refine the structure and assist in pattern formation. The retina develops from an
unpatterned layer of cells by differentiation. In this process, several superfluous cells form. Approximately 2000 cells are programmed to die to ensure the final pattern formation. Tanya Wolff showed that the retinal lattice is refined by the death that occurs between 35 and 55 hours after pupariation. Most of the cell death occurs between 35 to 40 hours but some continues until 55 hours post pupariation. The interommatidial cells undergo cell death tightening the lattice.

Programmed cell death in the optic lobe is seen in two phases, one at 48 hours and the second at 69 hours (Hofbauer and Campos-Ortega, 1990). Cell death in the lamina starts at pupariation approximately 15 to 20 hours after innervation. It is first seen in the posterior region, the region that has by now received innervations from the retina. It proceeds in a posterior to anterior direction, 3 to 5 cartridge rows at a time. Cell death reaches the anterior edge between 34.4 hours to 43.2 hours post pupariation. Cell death in optic lobe development takes place after innervation from receptor axons, but before synaptogenesis. During the first half of pupal development, degeneration moves towards the anterior rim. In sol mutants, excess degeneration is seen in the medulla at 17% post pupariation, that is 23.46 hours, peaks at 33% or 45.54 hours and ends at 46%.

The Blackpatch Mutation

The initial analysis of the Blackpatch mutation was conducted by Karen M. Duus and Jack R. Girton. Mutations at the Blackpatch locus cause death and degeneration of the retina and the optic lobe. Bpt is located between 92A6 to 92B1 on the third chromosome polytene band map, and 66.4 map units on the linkage map. Ten EMS-induced alleles of Blackpatch were isolated independently. These dominant mutations cause a black, necrotic patch on the eye. The alleles have variable penetrance and expressivity. The patch is usually restricted to the lower portion of the eye and in individuals with high expressivity, the patch is bigger but never reaches the edges. The patch is also bigger when the flies are
raised at a lower temperature. Mutants eclose with the eye patch in a light independent manner and it is not progressive with age. Sections through the patch show that the retina is collapsed, the lamina is reduced and often contains holes indicating degeneration, and the medulla is pulled out of position. The blackness seems to be a melanization of the dead and degenerating cells. Developmental analysis of this phenotype showed that the patch is seen as early as 72 hours into pupariation.

Somatic mosaic analysis indicates that the mutant action is not autonomous to the eye. The mutant retina in contact with normal optic lobes, or when isolated in culture, do not degenerate. Thus the requirement for retinal degeneration appears to be contact with a mutant optic lobe.

The Notch Locus and its Interaction with Blackpatch

Notch is a complex locus whose gene product is necessary at various stages in Drosophila development (Welshons, 1965; Artavanis-Tsakonas, 1988; Artavanis-Tsakonas, Delidakis and Fehon, 1991). The mutations at the Notch locus illustrate this complexity. Homozygous Notch embryos are inviable due to hypertrophy of the nervous system. Two classes of recessive mutations, alleles of Notch affect the wing and the eye respectively. These are the notchoid and facet alleles. There is also another dominant class of mutations, the Abruptex alleles that produce a wing vein phenotype. The name Notch refers to another heterozygous Notch phenotype that causes notched wings (Welshons, 1965).

The molecular structure shows that Notch is a transmembrane protein that is necessary for cell-cell signaling (Artavanis-Tsakonas, 1988; Greenwald and Rubin, 1992). In the context of the development of the embryonic nervous system, cells express Notch on the cell surface (Artavanis-Tsakonas, Delidakis and Fehon, 1991). This receptor protein is necessary for sending lateral inhibitory signals that prevent adjacent ectodermal cells from
adopting a neural cell fate. In the absence of inhibitory signals from *Notch*, cells adjacent to the presumptive neuroblasts also differentiate into neuroblasts and result in hypertrophy.

*Blackpatch* specifically shows an interaction with the *facet* (*fa*) alleles of *Notch*. There are six *facet* alleles, all of them, recessive. They are *fa, fa*, *fa*, *fa*, *fa*, and *fa* (Welshons, 1965). Flies bearing the *facet* alleles have extra secondary pigment cells in the retina (Cagan and Ready, 1989). This disrupts the precise lattice like structure and causes the eye to look rough. The *facet* alleles are associated with the insertion of a transposable element in the intron between exons B and C (Artavanis-Tsakonas, et al., 1984; Markopoulou, Welshons, and Artavanis-Tsakonas, 1989). There is some confusion regarding the naming of *fa*, *facet-strawberry*. It is included in the above list and said to have the insertion of a transposon like the other *facet* alleles. There is a different *facet-strawberry*, sometimes designated *fa*, that is associated with a 800 bp deletion close to the transcription initiation site.

A detailed description of the mutant phenotype of *facet-glossy* was provided by R. Cagan and D. Ready, 1989. In wild type flies, during pupal eye development, there are two primary pigment cells per ommatidium. They extend all the way around the cone cells and form a collar. Basally they are free of their foothold while the secondary pigment cells continue to have their basal foothold and participate to form the fenestrated membrane. In *facet-glossy* mutants, there are very few primary pigment cells, less than one per eight ommatidia. Instead there is an excess of secondary pigment cells. In an histological study of the adult visual system of *facet* mutants, they were shown to have defective optic lobe morphology (Markopoulou and Artavanis-Tsakonas, 1991). The implication of this study was that *Notch* is required for the maintenance of the optic lobe. In the mutants, there is reduced level of programmed cell death that accounts for supernumerary cells in the retina (Cagan and Ready, 1989). Experiments with a temperature sensitive *Notch* allele have shown that loss of *Notch* function during a specific time period in pupal development
mimics the phenotype of facet-glossy (Shellenbarger and Mohler, 1975; Shellenbarger and Mohler, 1978). The facet alleles have spatial and temporal specificity because the wild type protein is necessary in the development and patterning of the adult retina during the pupal stage of development.

The black patch phenotype is seen when the flies are mutant for facet and for Bpt (Duus, et al., 1992). In females, the patch is seen with the stronger facet alleles, fa^8 and fa^2. In males, along with these two, the patch is also seen in flies with fa^9 and fa^m. The mutant phenotype is necessary at a certain level of severity for the patch phenotype. Bpt does not show this interaction with any other allele of Notch. Double mutants of Bpt with the temperature sensitive allele of Notch were constructed (Duus, Welshons and Girton, 1992). The patch phenotype due to Bpt is seen when flies are exposed to a temperature regime that gives a strong facet-like eye phenotype (Girton, unpublished results). Towards the end of the temperature sensitive period, there is an overall reduction in the facet-glossy phenotype all over the eye. In double mutant N^t^1; Bpt individuals exposure to the restrictive temperature during this period results in progressively weaker facet phenotypes and a progressively smaller eye patch. Although the eye patch gets smaller with later exposures to the restrictive temperature, it always maintains its central-posterior location. The appearance of the patch along with the facet phenotype indicates its requirement for a tissue-specific reduction in Notch activity.

The Neurotrophic Hypothesis and a Possible Role for Blackpatch

In mammalian systems, there exists a mechanism to prevent neuronal cell death. Innervation of a tissue allows the elaboration of a trophic factor. This trophic factor is required to maintain the innervating axon. Absence of the trophic factor results in the death of the axon (Bothwell, 1995; Korsching, 1993). This interaction and dependence of the
axon on a specific factor that is secreted by a specific tissue to prevent cell death was conceived as the neurotrophic hypothesis.

Gene products of neurotrophic pathways are characterized by their requirement in specific cell types at a specific time in development. Nerve growth factor, NGF, the first neurotrophin that was discovered, is necessary for the maintenance of sympathetic ganglion neurons (Snider, 1994). They are also necessary for the survival of certain subpopulations of sensory neurons in the dorsal root ganglia and trigeminal ganglia. Cells of the peripheral nervous system do not require NGF but express two other types of neurotrophins, BDNF and neurotrophin-3 (NT-3). Experiments with knockouts of NGF in mice have shown differences in the time window for NGF dependence. The dorsal root ganglion cells have an early requirement for NGF and these cells are dead at the time of birth. The sympathetic ganglia, which have a later requirement, undergo massive cell death in the first few days of postnatal life.

Neurotrophic factors use cell signaling pathways. The NGF receptor is trkA, a tyrosine kinase receptor. Another related tyrosine protein kinase, trkB, is a receptor for BDNF and NT-3 (Klein et al., 1991). Receptor tyrosine kinases are a common motif for proteins in signal transduction pathways. By being in a phosphorylated or dephosphorylated state, signals are conveyed from upstream ligands to downstream molecules. Several other neuronal properties are regulated by neurotrophins, such as proliferation of precursors of neural crest cells (NT-3), synaptic rearrangement and dendritic arborization (NGF) (Bothwell, 1995; Lindvall, et al., 1994).

All the neurotrophins known so far prevent neuronal programmed cell death. The neurotrophic hypothesis is based on this last concept. The extent and timing of neuronal cell death is based on the influence of the target neurons (Bothwell, 1995). Innervation is competitive and those axons that make the connections receive the trophic factor and are able to survive. This modular approach accounts for a great deal of specificity in cell types
of target cells and innervating axons. Since it is now known that a very large number of neurons show a requirement for neurotrophic factors, a one to one approach is considered too simplistic. It is expected that complex interactions exist among neurotrophins that account for the complex diversity and simultaneous specificity of neurotrophic factors and target cell populations. In Drosophila, there is the Dtrk gene that encodes a tyrosine kinase receptor, closely related to the mammalian neurotrophic receptors (Pulido et al., 1992). The Trks are implicated in sending an anti-apoptotic signal on dimerization and phosphorylation (Bredeson and Rabizadeh, 1997).

Conclusion

The Bpt mutation is very significant among eye mutations in Drosophila. It is the only mutation described that has a focus in the lamina and a phenotype in the retina. Information about Bpt gene function will provide an insight into the development of the visual system. Neurotrophic factors have not been isolated in Drosophila yet. Bpt may be the first gene to be isolated and described in a neurotrophic pathway. It will open up another level of complexity in molecules regulating gene function. The interaction with Notch points to a function for facet-glossy in the lamina. This aspect of Notch function has never been addressed before. Bpt also has a function in early development indicating a vital function for Bpt. Analysis of Bpt function will provide a significant contribution towards the understanding of the nature of tissue interactions leading to organ system development.

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CHAPTER 2. CHARACTERIZATION OF THE ADULT VISUAL SYSTEM PHENOTYPE OF THE BLACKPATCH PHENOTYPE

A paper to be submitted to *Genetica*

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Abstract. Mutations at the Blackpatch (*Bpt*) locus cause death and degeneration in the visual system. To understand the role of *Bpt* in the visual system, the mutant phenotype was characterized. The experiments were designed to test two hypotheses for *Bpt* gene function: *Bpt* may be functioning in the visual transduction pathway or *Bpt* may be functioning in a neurotrophic pathway. To address the first hypothesis, the electrical activity of the fly eyes was measured. The results of the analyses show that *Bpt* does not function in the visual transduction process. To address the second hypothesis, the cell death phenotype due to the *Bpt* mutation was characterized. The characteristics of the Blackpatch phenotype are comparable to gene products in a neurotrophic pathway. Proteins in a neurotrophic pathway are localized spatially and temporally and they use cell signaling pathways. Mutations in neurotrophic pathways cause programmed cell death. Mutations in *Bpt* cause programmed cell death in a spatial and temporally specific manner. The Blackpatch phenotype is seen only in double mutants of *Bpt* and facet-glossy (*Notch*) suggesting that the two mutations are interacting. *Notch* is part of a signal transduction pathway and is used to send lateral inhibition signals to adjacent cells. With our evidence,
we suggest that $Bpt$ may be functioning in a neurotrophic pathway along with $Notch$ to maintain laminar cells and prevent cell death.

*Key words. Drosophila melanogaster, electroretinograms, apoptosis, retina-lamina*

**Introduction**

A fundamental tenet of developmental genetics is that the characterization of the phenotype given by a mutant allele of a gene provides information about the normal function of the wild type allele. As our objective is to understand the normal function of $Blackpatch$ in the development of the visual system, we have characterized the effect of $Bpt$ mutant alleles on the structure and function of the visual system. $Blackpatch$ mutations cause extraordinary amounts of cell death in the developing visual system. The normal function of this gene is vital for the correct development and maintenance of cells in certain regions of the adult visual system (Duus, Welshons and Girton, 1992). We have attempted to use our results to test the predictions of two models for $Bpt$ action in nervous system development; models based on the function of other genes that affect the adult visual system. A key to this type of analysis is to identify important aspects of the mutant phenotype and to examine models that may explain how this phenotype is produced. For $Bpt$ we have focused on models that can explain the induction of cell death. There are two such models, based on two types of mutations that are known to cause excessive cell death in the eye and optic lobe of the brain in *Drosophila* and other animals.

Our first hypothesis was that $Bpt$ is necessary for the function of the adult visual transduction pathway and that $Bpt$ mutations cause cell death by altering this system. Mutations of other genes that are known to be in this pathway cause cell death and degeneration in the eyes and optic lobes. These genes play an important role in light processing and the mutations cause death by affecting the electrical activity of the eye.
Examples of this type of mutation are the rdg mutants (Harris and Stark, 1977). Flies with rdg mutations have altered electrical activity in the eye and optic lobe, and have progressive degeneration in the eye. It is possible that Bpt may function in the visual transduction pathway, and that Bpt -induced cell death results from abnormal function of this system. If so, then flies with Bpt mutations should also show other characteristics of mutations affecting the visual transduction system, notably an altered ability to respond to light.

Electroretinograms (ERGs) are a visual measure of the electrical activity in the retina and optic lobe of the brain in response to light, and abnormal function of the visual transduction system can be detected by analyzing the shape and intensity of electroretinograms (Pak, Grossfield and Arnold, 1970). All 10 different Bpt mutant alleles were analyzed for their electroretinograms to determine whether Bpt mutant flies had abnormally functioning visual transduction systems. The questions addressed by these experiments were: a) Does Bpt mutation affect the electrical activity of the cells that have the mutant genotype but are not part of the patch, b) are the electrical activities of the cells in the retina and/or the lamina altered, c) what is the effect of the patch size on the electrical activity of the eye, and d) what are the effects of the different alleles of Bpt on the electroretinograms.

The alternate hypothesis for Bpt function is that this gene has an essential function during the development of the visual system. In mammalian systems, neurotrophic factors play a significant role in the development and maintenance of the nervous system (Bothwell, 1995). Neurotrophic factors are elaborated by neurons or their targets and are necessary for establishing and maintaining the complex connections in the nervous system (Snider, 1994). In addition, neurotrophic factors act as mitogenic agents for neuronal stem cells and glia (Bothwell, 1995; Lindvall et al., 1994). The important characteristics of neurotrophic factors are: a) they show very specific spatial and temporal action, controlling the growth and/or survival of specific sets of neural cells, b) they are involved in cell-cell
signaling mechanisms, and c) mutations in genes in neurotrophic pathways cause
programmed cell death (Korschning, 1993). At present, no genes for neurotrophic factors
have been proven to exist in Drosophila. Dtrk, a Drosophila gene with high homology to
the trk family of mammalian neurontrophin receptors has been identified (Pulido, 1992).
This suggests that there may exist neurontrophin genes and their pathways in Drosophila as
well. Some characteristics of Bpt mutations resemble mutations of genes in mammalian
neurotrophic pathways.

In this chapter, the results of several experiments are presented that give a detailed
description of the effects of Blackpatch mutations on the function of the visual system and
of the Bpt-induced cell death in the eye and optic lobes. These results have provided a
great deal of information about the spatial and temporal specificity of Bpt function. These
results are discussed in light of the predictions of the two models for Bpt action. We
believe the results support the hypothesis that Bpt is a vital gene in a neurotrophic pathway
active in the development of the adult optic lobe. Its gene product may be necessary for the
correct development and maintenance of the visual system in Drosophila.

Materials and Methods

Genetic stocks

The eye patch phenotype is seen when the flies have a double mutant background of
Bpt and facet. All the Bpt stocks in the laboratory are maintained in a white-apricot, facet-
glossy background, i.e., as w^fa^; Bpt. There are 10 Bpt alleles designated, Bpt^, Bpt^, Bpt^, Bpt^,
Bpt^, Bpt^, Bpt^, Bpt^, Bpt^, and Bpt^'. The homozygous lethal alleles, Bpt^, Bpt^, and Bpt^' are maintained on a TM6, Sb balancer. The others are maintained as
homozygotes. Two types of control genotypes were used in our analyses. These are
yellow, white, singed (y w sn), and white-apricot, facet-glossy (w^fa^). The white
mutation in y w sn affects the pigmentation and these flies have white eyes. White eyed
flies are commonly used as controls in the place of red eyed, wild-type flies in analyses of electrical activity in the fly eye. The lack of red pigment in the y w sn flies prevents light adaptation (De-Mao Chen, personal communication). Therefore, y w sn flies were used to obtain the standard (comparable to a wild-type) ERG. The w^fa^ flies were the second type of control genotype to observe the effect of the background, if any were used. Twenty five males and females from each stock (controls and Bpt') were mated and among the progeny, fresh flies of both sexes were collected at random and aged 3 days before they were used to measure electroretinograms. To maintain the appearance of the patch in the eyes of Bpt' flies, they were regularly crossed back to Bpt flies that are constantly selected for big patches.

For analyzing the cell death phenotype in the eye, the genetic stocks used were w^fa^, Wfa*/Wfa^: Bpt'/Bpt', and Wfa^+/+. Bpt'/TM 2(6). The strain, Wfa^+/+; Bpt'/TM 2(6) was constructed as follows:

w^fa^/Y; Bpt'/Bpt' X +/-; TM2/TM6

The progeny from these flies were heterozygous for Wfa^ and Bpt'. Since females have higher penetrance and expressivity of the patch, female larvae with the genotype, Wfa^+/+: Bpt'/TM 2(6) were used for the analyses. The control genotype was Wfa^.

Preparing the fly for measuring electroretinograms

The black eye patch phenotype has variable penetrance and expressivity. A fly of the genotype, Wfa^; Bpt may not have a visible patch (= 0% patch) and its sibling may have a very big patch (=85% patch). The flies were divided into 3 classes, the 0-10% class, 45-55% class and 75-85% class. These 3 classes represent 3 sizes of the patch without any overlap. The size of the black patch was measured by expressing it as a percentage measure of the total eye. This was calculated by drawing a diagram of the mutant fly eye under 10x magnification of the dissecting scope, on a grid containing 225 squares, (Figure 1). This number was converted into a percentage. Accordingly, flies
Figure 1. A diagram of the patch was drawn on a grid of 225 squares and converted into a percentage value.
belong to a class with 0 to 5% patch, 45 to 55% patch or 75 to 85% patch. Flies were collected from the bottle in the anaesthetizer and examined to ensure that the condition of the fly and the eye was healthy. For flies with patches, the size of the patch was determined for each eye and the fly was assigned to a particular size class. The flies were placed in a pattern on the coverslip to enable each fly to receive the light stimulus. A drop of nail polish was placed on the legs and the wings to adhere the fly to the cover slip.

The coverslip with the flies was placed on a block of agar in 0.7% NaCl made up in a plastic box of 4" x 3". Small and thin rectangular pieces of agar, about 1mm x 2mm, were sliced using a razor blade. These slivers were placed to span the body of the fly and the agar block (Figure 2). The agar was kept moist during the experiment by spraying 0.7% saline. Each coverslip contained a “batch” of flies. Every batch always contained the following flies in the same sequence. The first two flies starting from the top left hand corner of the coverslip were y w sn flies, controls used to ensure that the electrical circuit is functioning correctly. The third fly was a w^t_f^t fly, the second control genotype to measure the effect of the facet-glossy background. Following this were 2 Bpt flies. These 5 flies were on the left hand side of the cover slip. On the bottom edge of the cover slip were 3 more Bpt flies followed by the last fly that was always a y w sn fly, again the control genotype to ensure that the electrical circuit is still functioning correctly. For the w^t_f^t; Bpt^t genotype, a random assortment of flies with different sized patches were used in each batch. In the batches for w "fa^t; Bpt^t, w "fa^t; Bpt^t, w "fa^t; Bpt,^t and w "fa^t; Bpt^t" genotypes, the ERG was measured from the mutant flies and w^t_f^t (the background control genotype) alternately.

The electrodes used to measure electroretinograms

A cotton wick electrode was used to measure the electroretinogram from the area of the patch. The patch on the eye is a hard scab like condition and a fragile glass electrode cannot penetrate the eye in the region of the patch. Glass electrodes were made, filled with
Figure 2. The diagram shows a view of a fly on a cover slip placed on a block of agar. An electrical connection is set up between the fly and the block using a thin piece of agar.
Reference electrode

Agar piece

Angle of the fly

Block of agar

Cover slip
saline and a single fiber of cotton was inserted into the tip. This type of electrode was placed on the patch. A drop of saline was placed between the cotton wick and the eye to allow conduction of electric current.

To measure electroretinograms from the area adjacent to the patch, thin glass electrodes were used. The region of the eye adjacent to the patch is genotypically mutant but does not have the patch. Glass recording electrodes with 1μm diameter at the tip were made. The electrode is filled with saline, fitted into the micromanipulator and inserted into the area adjacent to the patch. Recording electrodes were made using glass capillaries, Kwik-Fil Thin-Wall Single-barrel Borosilicate (type 7740) glass tubing with filament, No. TW100F-3, World Precision Instruments, INC. (Sarasota, FL) with a Brown-Flaming Micropipette Puller, Model P-77, Sutter Instrument Company. Glass electrodes with 1μm diameter and a resistance of 1 to 10 MΩ are considered optimum to insert into fly eyes to measure electroretinograms. Several parameters like the heat setting, gas flow and trip point have to be adjusted to get the desired micropipettes (glass electrodes).

Two electrodes can be made at one time and each electrode was measured for tip length and resistance. The resistance was measured on the oscilloscope. The glass micropipette (recording electrode) was filled with 0.7% NaCl using a thin syringe. This was inserted into the micromanipulator and placed in a petridish filled with 0.7% NaCl. The reference electrode was also introduced into the same dish to complete the circuit. In this experiment, the gas flow was set at 200, trip point was 0.05 and the heat setting at 198. The results for two sets of electrodes is given in Table 1. The resistances are higher than desired and they were lowered by changing the the gas flow setting to 225. The resistances of eighteen electrodes was between 4 MΩ to 11.5 MΩ and the average is shown in Table 2. For the electrodes used to measure electroretinograms, the resistance was measured at the beginning and ending of the experiment.

The electrode holder was prepared such that glass electrodes could be inserted in
Table 1. Summary table of resistance measurements (mV) of the recording electrodes with the gas flow set at 200.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Tip lt. (mm)</th>
<th>mV/D</th>
<th>#D</th>
<th>mV</th>
<th>Ω</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>3</td>
<td>10</td>
<td>3.0</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>1b</td>
<td>3</td>
<td>50</td>
<td>4.6</td>
<td>220</td>
<td>22</td>
</tr>
<tr>
<td>2a</td>
<td>4</td>
<td>50</td>
<td>4.6</td>
<td>230</td>
<td>23</td>
</tr>
<tr>
<td>2b</td>
<td>4</td>
<td>50</td>
<td>3.4</td>
<td>170</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 2. Summary table of resistance measurements (mV) of eighteen electrodes with gas flow set at 225.

<table>
<thead>
<tr>
<th>Tip lt.</th>
<th>mV/D</th>
<th>#D</th>
<th>mV</th>
<th>Ω</th>
<th>ΣX-X</th>
<th>S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.27</td>
<td>50</td>
<td>1.45</td>
<td>105</td>
<td>7.25</td>
<td>32</td>
<td>7.76</td>
</tr>
</tbody>
</table>

and taken out with minimum disturbance to the entire set-up. The glass electrode was injected with 0.7% NaCl and inserted into a one inch electrode holder with a metal prong at the other end that fitted into a larger 6 inch glass holder. A metal pin was soldered to the side of the large holder such that the wire leading to the Dagan pre-amplifier could be inserted into the pin. The large holder with the wire was held in the micro-manipulator. The large holder with the wire could be left in the micro-manipulator for months and this reduced the disturbance to the entire set-up.

A silver reference electrode was used. This electrode is necessary to ground all the extraneous electrical activity from the body of the fly. The silver reference electrode was inserted directly into the agar block. As described above, the flies on the coverslip were placed on this agar block and electrical connection between the flies and the agar was achieved by thin pieces of agar.
The components of the rig used to measure electroretinograms

The electrical set up to measure the ERGs consisted of the recording and reference electrodes, the Dagan pre-amplifier and the oscilloscope. The signals from the fly eye was amplified 10x by a preamplifier, Dagan corporation (Minneapolis, MN) and then displayed on the screen of an oscilloscope. The signals picked up by the reference electrode was fed into the pre-amplifier and grounded.

A fiber optic illuminator provided a white light stimulus that was focused onto the eyes by using a mirror and a convex lens. The wavelength of the light stimulus was controlled by using wavelength filters of 470 nm and 568 nm. Neutral density filters were used to control the intensity of the light stimulus. Table 3 shows the % absorbance and % transmittance of the filters at 470 nm and 568 nm.

Table 3. The % absorbance and % transmittance values of the nine neutral density filters used in measuring electroretinograms

<table>
<thead>
<tr>
<th></th>
<th>absorbance</th>
<th>transmittance</th>
<th>absorbance</th>
<th>transmittance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.293</td>
<td>51.0</td>
<td>0.301</td>
<td>51.2</td>
</tr>
<tr>
<td>2</td>
<td>0.290</td>
<td>51.2</td>
<td>0.297</td>
<td>50.7</td>
</tr>
<tr>
<td>3</td>
<td>0.288</td>
<td>51.6</td>
<td>0.293</td>
<td>51.2</td>
</tr>
<tr>
<td>4</td>
<td>0.472</td>
<td>33.8</td>
<td>0.477</td>
<td>33.4</td>
</tr>
<tr>
<td>5</td>
<td>0.461</td>
<td>34.8</td>
<td>0.462</td>
<td>34.3</td>
</tr>
<tr>
<td>6</td>
<td>0.474</td>
<td>33.4</td>
<td>0.481</td>
<td>33.5</td>
</tr>
<tr>
<td>7</td>
<td>0.984</td>
<td>10.3</td>
<td>0.960</td>
<td>10.6</td>
</tr>
<tr>
<td>8</td>
<td>0.978</td>
<td>10.6</td>
<td>0.971</td>
<td>11.1</td>
</tr>
<tr>
<td>9</td>
<td>0.970</td>
<td>10.6</td>
<td>0.995</td>
<td>10.6</td>
</tr>
</tbody>
</table>
Three filters with an absorbance value of 0.3 (1, 2, 3) were placed in a series followed by three filters with an absorbance value of 0.5 (4, 5, 6) and three filters with an absorbance value of 1 (7, 8, 9). Electroretinograms were measured at four different intensities. The lowest light intensity (a) was achieved by placing all the nine filters (1 to 9) in the path of light. Intensity (b) was achieved by placing six filters (1 to 6) and intensity (c) was achieved by placing (1 to 3) in the path of light. Highest light intensity was obtained when no neutral density filters were used. The filters were placed between the two plastic sides of a slide holder. This helped to hold the filters in place. A plexiglass box was constructed with holes on either side to allow for the passage of light. Slats present in the middle of the box held the slide holders. The construction allowed the necessary filters to be in the path of light and others could be moved out of the path of light. The duration of the light stimulus was controlled by a camera shutter. The exposure was set to 1 second to ensure that the duration of the light stimulus was consistent throughout the experiments. A simplified diagram of the set-up is shown in Figure 3. The set-up described so far was placed on a heavy metal table inside a Faraday cage. A sheet of metal wire fixed to a wooden frame was inserted to divide the cage into 2 chambers. The larger right half housed the microscope, the mirror, convex lens and the micro-manipulator. The shutter and the box of filters were placed in the left half. The Faraday cage and the divider were covered by black paper to prevent the interior of the cage from receiving extra light. The front of the cage had a black curtain that could be parted at will. It could be closed completely during the dark adaptation.

**Method of data collection**

Since the oscilloscope trace moves at different speeds, the ERG can be measured at 1 second/Division (1s/D), 0.5s/D or 0.2s/D. In the figure, one division is seen as one large square with twenty five small squares. The oscilloscope is more sensitive at higher speeds and ERGs of very low amplitudes are measured at high speeds. The amplitude of the ERG
Figure 3. This diagram shows the set up used to measure electroretinograms. The recording electrode was inserted into the fly eye and electrical signals were converted to a visual trace (ERG) on the oscilloscope. The reference electrode inserted into the block of agar grounded out the extraneous electrical activity. The light stimulus passed through a series of wavelength and neutral density filters.
is measured as mV/Division. It can be measured at 10mV/Division or 20mV/D or 100mV/D. The oscilloscope is most sensitive at 10mV/D and the sensitivity of the oscilloscope can be calibrated as necessary. To report the results, all the ERGs were converted to measurements at 100mV/D. Data regarding electroretinograms were collected by tracing the figure on the oscilloscope screen onto a transparent graph sheet. The speed and the mV/Division setting was noted.

Measuring electroretinograms from pupae

Pupae older than 70 hours were used in this study. The puparium was removed using two pairs of forceps. The pupae were attached to the coverslip either with hot wax or nail polish. Each pupa was placed alternately with a y w sn adult fly. The pupae have a pupal cuticle which is a thin, transparent covering over the entire body including the eye. For each pupa, this pupal cuticle was removed from the region of the eye using a pair of fine forceps. The remaining procedure for measuring ERGs was the same.

Collection of pupae for trypan blue staining

Fifty males and females were used in a bottle that was sub-cultured every 3 days to prevent crowding. The flies were always raised at a constant temperature, 25°C. Prior to collection, the bottles were cleaned of all pupae with a wet brush and these pupae were used as a control batch to analyze for the size of their patches. After the initial cleaning, white prepupae were collected every 2 hours. Every batch was collected separately in a glass vial with no food. The pupae were placed on the sides of the vial. They were allowed to develop for varying lengths of time prior to dissection. Female flies with the Bpt mutation have higher penetrance and expressivity of the patch. For the experiments to determine the staining pattern and timing of the retina, female larvae were selected and allowed to develop separately into prepupae.

Microdissection and staining of the pupal visual system

The visual system consists of the retina and the underlying optic lobes. It was
dissected from the pupae and stained with trypan blue. Trypan blue is a vital dye that is taken up only by dead cells. The thin lid of 60mm plastic petridishes was used for the dissections. The thinness helps because it holds much less solution since too much solution obscures the vision. The pupal case was opened and the pupa was decapitated using a pair of forceps. A sharp pin was filed to make a flat cutting edge and this was fitted into a holder. This instrument along with a pair of forceps was used to dissect the head open and reveal the retina and optic lobe complexes. The head tissue around the complexes was retained to avoid damaging the edges of the retina and to be able to pick up the complex with a pair of forceps. The small petridish was place in a larger dish filled with ice to keep the tissue cold. Approximately 5 to 7 pupae were dissected over a period of twenty minutes spanning the mid-point of the collection.

Two mls of a saturated solution of trypan blue was diluted to 10mls. Approximately, 500 µl of this diluted solution was used for each batch. The opened head capsules from five to seven pupae were transferred to a transparent glass dish with several depressions in it. Each batch was placed in one depression and stained together for 10 min. At the end of 10 minutes, about 1 ml of 0.75% NaCl was added to fill the entire depression. This procedure dilutes the stain and also allows the experimenter to be able to see the complexes. The complexes were washed by placing them in a new depression slide with fresh 0.75% saline. The tissue had to be specially prepared for close observation. A thin edged razor and a pair of very fine forceps were used to remove the tissue around the visual complex. Some granules of trypan blue sticking to the tissue were removed.

Method of data collection

For every complex that was stained, a quick diagram of the retina and lamina was made in the notebook and the location of the stain was entered and described. The tissue was examined for being exposed to stain which is seen as a pinkish hue in the medulla and for any injury sustained during dissection. For the complexes stained in the retina, the
patch of stain was drawn on the template that has already been explained. Data was also collected regarding the patch size of the siblings of the dissected and stained pupae.

The visual system has a very three dimensional construction because the retina sits like a mushroom on the end of a stem. The lamina is a very thin layer of tissue that lies inside the concavity of the retina. The stain in the lamina was visible through the retina or could be viewed by lifting and pushing the retina to one side. The tissue was placed between two coverslips on a slide with a third coverslip on the top. The extra space allowed the surface of the retina to be seen without distortion and could be photographed under the compound microscope.

Cell death analyses using TUNEL

TUNEL is an abbreviation for terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling. This is a standard technique used to label apoptotic cells. White prepupae of \( w^{fa}; Bpt \) genotype were collected and dissected as described. The dissected pupae were fixed for 25 min. by using heptane and formaldehyde followed by washes with methanol and ethanol. They were rinsed six times in phosphate-buffered saline that contained 3N Triton-X and once in TdT buffer, Boehringer Mannheim (Indianapolis, IN). The complexes were isolated and trimmed for better viewing prior to TUNEL. Apoptotic cells were identified by incubation in Label solution from an In Situ Cell Death Detection Kit, AP, No. 1684809, Boehringer Mannheim (Indianapolis, IN) for 3 hrs. at 37 °C. This was followed by incubation in Alkaline Phosphatase converter for 1 hr and then treated with NBT and X-Phosphatase for 10 min in a color reaction.

Results

Electroretinograms can be measured using cotton wick electrodes and glass electrodes.

The patch on the eye caused by \( Bpt \) mutations is very hard and scab-like and a glass electrode cannot be inserted through such a patch. Cotton wick electrodes were used to
measure electrical activity directly from the surface of the patches. The disadvantages of cotton wick electrodes are that their preparation is tedious. The electrical connection between the cotton wick and the surface of the eye is maintained by a single drop of saline that must be carefully placed between the two using a syringe. For most of the remaining experiments, glass electrodes were preferred because of their ease of preparation. The insertion of the electrode into the eye easily provides the physical connection necessary for electrical conductivity. Though invasive, the loss of a few cells in the eye does not affect the total electrical activity of the 800 ommatidia of the compound eye.

The electroretinograms of a fly with y w sn genotype and a fly of the w^{fa*} genotype were measured using a cotton wick electrode. The same flies were used for subsequent measurements with the glass electrode. Eight measurements were taken from each fly. Figure 4 shows the ERG of a wild type Ore R fly measured using a cotton wick electrode. The ERG was measured at 0.1V/D and at a speed of 1 sec/D. The amplitude is 10mV (100/10 due to 10x amplification). The laminar and retinal components of the electroretinograms are present.

*ERGs of a wild type fly were measured at three different regions of the eye.*

*Blackpatch* flies have a patch in the central-posterior region of the eye and in such flies, ERGs can only be measured from the anterior regions. ERGs were measured at different locations of the eye in a control genotype to ensure that the electrical activity could be consistently measured from the different regions. The three regions where the ERG was measured are shown in the diagram in Figure 5. Since the same eye was used to measure ERGs three times, damage to the eye was prevented by using a cotton wick electrode. Previously, both glass and cotton wick recording electrodes were shown to successfully measure ERGs and the ERGs measured by the two methods were comparable. The ERGs were measured at 0.1V/D and at 1sec/D. The amplitude of the ERGs from the 3 regions of one eye are 18mV from region 1, 18mV from region 2, and 18mV from region 3,
Figure 4. Electroretinogram of Ore R using a cotton wick electrode measured at 0.1 V/D and speed of 1 sec/D. Stimulus lasted 1 sec and is shown as a white bar. The laminar transients are indicated by white arrows. The arrowhead points to the retinal negative.
Electrical activity can be measured from any of the three regions in the eye. Regions 2 and 3 represent the usual location of the black patch in mutant flies. Region 1 represents the usual location of the insertion of the recording electrode in succeeding experiments.

The electroretinograms of control genotypes

The \( y w sn \) genotype was used to obtain normal ERGs which would be the equivalent of the wild type ERG. Figure 7 shows a sample ERG of a \( y w sn \) fly measured at 0.1V/D and at 1sec/D. The amplitude is 16.5mV (165/10 for 10x amplification). The shape of the ERG is consistent with the published wild-type results. The on and off laminar transients and the sustained negative are present. This control genotype was always used later to ensure that the experimental set-up was working correctly. The average amplitudes of the ERGs (mV) at two wavelengths, 470nm and 568nm at four intensities, a, b, c, and d are given in Table 4. The ERGs measured at different wavelengths and intensities had the laminar and retinal components.

<table>
<thead>
<tr>
<th></th>
<th>470nm</th>
<th></th>
<th>568nm</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>d</td>
</tr>
<tr>
<td>( n )=22</td>
<td>3.7</td>
<td>4.0</td>
<td>4.0</td>
<td>3.7</td>
</tr>
</tbody>
</table>

The second control genotype was \( white-apricot, facet-glossy \). The ERGs of \( w^{fa^r} \) flies have laminar on and off transients and the sustained retinal negative. Figure 8 (A) shows an ERG from a \( y w sn \) fly and (B) shows an ERG from a \( w^{fa^r} \) fly measured at 0.1V/D and at 1sec/D. Both the ERGs show the presence of laminar transients and retinal transients...
Figure 5. The three different regions of placement of the cotton wick electrode in a y w sn fly. Region 1 in the anterior area of the eye is the usual location for the insertion of the recording electrode. Regions 2 and 3 show the usual location of the patch.

Figure 6. Electroretinograms from the three regions are shown. The first row shows the ERGs from region 1, the middle row from region 2, and the last row from region 3. They were measured at 100mV/D and speed of 1sec/D. Simulus lasted for 1sec and is shown by a white bar.
Figure 7. Representative electroretinogram of a $yw sn$ fly measured at 100mV/D and speed of 1sec/D. The stimulus lasted 1sec and is shown as a black bar.

Figure 8. Comparison of the ERGs of the two control genotypes, $yw sn$ (A) and $w^{fa} (B)$. They were measured at 100mV/D and a speed of 1sec/D. The “on” and “off” of the stimulus that lasted for 1sec is indicated.
negative. The amplitude of the ERG at (A) is 12mV and the amplitude of the ERG at (B) is 10mV (divided by 10 for amplification). The average amplitudes of the ERGs of 14 w^{fa^n} flies at two wavelengths and four intensities is shown in Table 5. The ERGs of w^{fa^n} flies always showed laminar transients and retinal negatives.

Table 5. The average amplitudes (mV) of the ERGs of 14 w^{fa^n} flies at two wavelengths and four intensities.

<table>
<thead>
<tr>
<th></th>
<th>470nm</th>
<th>568nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>2.8</td>
<td>3.2</td>
</tr>
<tr>
<td>b</td>
<td>3.4</td>
<td>3.5</td>
</tr>
<tr>
<td>c</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>d</td>
<td>3.3</td>
<td>3.4</td>
</tr>
</tbody>
</table>

The amplitude and shape of this ERG can be used as a standard for comparison with the Bpt mutants. It is also a good control to confirm that the electrical circuit is working. The background mutations in our Bpt stocks, that is the white-apricot and the facet-glossy, do not affect the shape and amplitude of the electroretinograms. The facet-glossy mutation which affects the structure of the retina does not affect its response to light at these two wavelengths (470nm and 568nm).

Electroretinograms of Blackpatch'flies

Two different approaches were taken to determine whether the cells in a patch retain any electrical response to light. First, cotton wick electrodes were used to measure ERG’s from the surface of the patch. No response to light was recorded. Second, glass electrodes were carefully inserted such that their tips were lying underneath the patch. The position of the electrode is shown in a diagram in Figure 9. In this position, no electroretinogram was recorded from the fly. These results confirm that the region of the
Figure 9. This diagram shows the location of insertion of the recording electrode, underneath the patch.
Electrode at junction

Anterior

Ventral

Black patch on the eye
patch cannot perceive and respond to light. The region underneath the patch does not process light either.

*Blackpatch flies have electroretinograms that are coincident with the control*

The shape and amplitude of the ERGs of flies with 0 to 10% patches were measured. These flies are genetically \( fa^c; Bpt^1 \) but have only a small or no patch. We report two important findings from this experiment. Regardless of the mutant genotype, these eyes are electrically active and repeatable ERGs were measured from these flies. The ERGs of these flies have the laminar transients and retinal negative as in the control. The control in Figure 10(A) was measured at 0.1V/D and at a speed of 1sec/D and has an amplitude of 16.5 mV. The ERG from a \( fa^c; Bpt^1 \) with 0% patch shown in Figure 10(B) was measured at 0.1V/D at a speed of 1 sec and has an amplitude of 14.5 mV (amplitudes adjusted for 10x amplification). The average amplitudes of 12 \( w^{fa^c}; Bpt^1 \) flies with 0 to 10% patches at two wavelengths and four intensities is shown in Table 6 in mV.

<table>
<thead>
<tr>
<th></th>
<th>470nm</th>
<th></th>
<th>568nm</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>3.7</td>
<td>4.2</td>
<td>4.4</td>
<td>4.4</td>
</tr>
</tbody>
</table>

*Electroretinograms show reduction in amplitude as patch size increases*

To measure the effect of the patch, the ERG was measured from \( Bpt \) flies with different sized patches. A control \( y w sn \) fly has an ERG of 0.09mV, Figure 11(A), while a \( w^{fa^c}; Bpt^1 \) fly with 55% patch has an ERG with an amplitude of 0.035mV, Figure 11(B). A fly with 71% patch has an ERG with an amplitude of 0.03mV, Figure 11(C).
Figure 10. Comparison of ERGs of a y w sn fly (A) and a w^fa'; Bp^f fly with 0° patch (B). ERGs were measured at 100mV/D and at 1sec/D. The stimulus lasted for 1 sec and is shown as a black bar underneath the trace.
Figure 11. Electroretinograms of y w sn fly (A), w"fa" Bpt' fly with 55% patch (B), and a w"fa" Bpt' fly with 71% patch (C) were measured at 10mV/D and a speed of 1/sec/D. The stimulus lasted for 1 sec and the "on" and "off" is indicated in all 3 traces.
Figure 11, the ERGs were measured at 10mV/D and 1sec/D. The flies with moderate sized patches and large patches have amplitudes that are much lesser than the y w sn flies. The flies were divided into three size classes depending upon the size of the patch. The average amplitudes of flies from the 45 to 55% class is given below in Table 7.

Table 7. Average amplitudes (mV) of the ERGs of w^fa^; Bpt^ flies with 45 to 55% patches.

<table>
<thead>
<tr>
<th></th>
<th>470nm</th>
<th></th>
<th>568nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>b</td>
<td>c</td>
<td>d</td>
</tr>
<tr>
<td>n=7</td>
<td>n=7</td>
<td>n=7</td>
<td>n=9</td>
</tr>
<tr>
<td>1.1</td>
<td>1.4</td>
<td>1.6</td>
<td>1.4</td>
</tr>
</tbody>
</table>

The average amplitudes of the ERGs of eleven flies of the 75 to 85% class is given below in Table 8. For several flies, the amplitude was extremely low and almost negligible at lower intensities. At the highest intensity of light stimulus, a comparison of the average amplitudes of the different genotypes is given below in Table 9.

The components of the electroretinograms are more obvious at slower speeds.

The speed of the trace can be changed to increase the sensitivity of the recording.

The components of the electroretinogram are more obvious when the speed is reduced.

Table 8. Average amplitudes (mV) of the ERGs of w^fa^; Bpt^ flies with 75 to 85% patches.

<table>
<thead>
<tr>
<th></th>
<th>470nm</th>
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</thead>
<tbody>
<tr>
<td>a</td>
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<tr>
<td>1.1</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>568nm</td>
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<td>b</td>
<td>c</td>
</tr>
<tr>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
<td>n=13</td>
</tr>
<tr>
<td>1.3</td>
<td>1.7</td>
<td>0.9</td>
<td></td>
</tr>
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</table>
Table 9. Average amplitudes (mV) of different genotypes at the highest light intensity, measured at 0.1V/D

<table>
<thead>
<tr>
<th>Genotype</th>
<th>470nm</th>
<th>568nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>y w sn</td>
<td>3.7</td>
<td>4.2</td>
</tr>
<tr>
<td>w&quot;fa&quot;</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td>w&quot;fa&quot;; Bpt' (0 to 5%)</td>
<td>4.4</td>
<td>4.7</td>
</tr>
<tr>
<td>w&quot;fa&quot;; Bpt' (45 to 55%)</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>w&quot;fa&quot;; Bpt' (75 to 85%)</td>
<td>1.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

In Figure 12(A), the ERG of a y w sn fly was recorded at 50mV/D and at a speed of 1 sec/D. The ERG from the same fly is shown in Figure 12(B) when recorded at 0.2sec/D. In both traces, the amplitude is 2mV (40mV/ 10 since the trace is 10x amplified and divided by 2 since all the amplitudes are presented for 100mV/D).

Electroretinograms from Bpt fly eyes with large patches have laminar transients

The electroretinograms of all the flies, regardless of the size of the patch always showed the laminar transients and the retinal negative. Even in flies with a very large patch (85%), where the total electrical activity is greatly reduced, the ERG showed distinct on and off peaks and the sustained retinal response. This result was true for all measurements at both wavelengths and all intensities. Figure 13 shows the results.

Electroretinograms of flies with different Blackpatch alleles

The ERGs of a varying percentage of flies with other Bpt alleles do not have laminar activity. Characterizing a mutant phenotype in a series of alleles is necessary to understand any allele-specific variations that might exist. We were specifically interested in the effect of Bpt alleles on the retinal and laminar components of the electroretinogram. To determine this, all the ten alleles of Bpt were characterized for the shape of the ERG (Table 10). Flies with different alleles of Bpt sometimes showed a dramatic difference in the
Figure 12. ERGs of one y w sn fly. The trace at (A) was measured at a speed of 1 sec/D and the trace at (B) was measured at 0.2 sec/D. The amplitude scale is set to 100 mV/D. The stimulus lasted for 1 sec and the "on" and "off" is indicated.

Figure 13. Representative electroretinograms of w"fa" Bpt' flies from 45 to 55% class (A) and 75 to 85% class (B). ERGs were measured at 50 mV/D with a stimulus of 1 sec that is shown as a black bar. (A) was recorded at a speed of 0.5 sec/D and (B) at 0.2 sec/D.
Table 10. A variable percentage of flies with different alleles of \( w^\text{fa}^{\text{b}}; Bpt \) have the laminar peaks indicating electrical activity in the lamina.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Total number of flies whose ERG was measured</th>
<th>Percentage of flies with laminar peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Bpt' )</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>( Bpt^2 )</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>( Bpt^1 )</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>( Bpt^4 )</td>
<td>9</td>
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</tr>
<tr>
<td>( Bpt^5 )</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>( Bpt^6 )</td>
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<td>0</td>
</tr>
<tr>
<td>( Bpt' )</td>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>( Bpt^7 )</td>
<td>4</td>
<td>75</td>
</tr>
<tr>
<td>( Bpt^8 )</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>( Bpt^9 )</td>
<td>13</td>
<td>46</td>
</tr>
</tbody>
</table>

shape of their ERGs. For all of them, the retinal component was always normal. The laminar component was either present as in the normal or was missing completely with both the peaks absent. The elimination of peaks appeared to be an all or nothing event. Figure 14 shows ERGs at four different intensities of a \( w^\text{fa}^{\text{b}}; Bpt \) fly. The laminar transients are absent. Based on the shape of the ERG, the \( Bpt \) alleles can be divided into three groups. \( Bpt' \) and \( Bpt' \) flies have both the retinal negative and laminar peaks all the time (100%). A second group of alleles including \( Bpt^2, Bpt^4, Bpt^6 \), and \( Bpt^8 \) have only the retinal negative and never have the laminar peaks (0%). A third group of alleles showed variability in the presence and absence of the laminar peaks. \( Bpt' \) had laminar peaks only 13% of the time and \( Bpt' \) had laminar peaks 15% of the time. While 75% of the sample of \( Bpt^6 \) flies had laminar peaks, only 46% of \( Bpt' \) had them. We can describe an
Figure 14. ERGs of a w^{fa}: Bpf^{a} fly recorded at 100mV/D and speed of 1 sec/D. The stimulus lasted 1 sec and is shown as a white bar.
allelic series with regard to the percentage distribution of flies which have laminar peaks, 
\( Bpt^t = Bpt^r > Bpt^a > Bpt^z > Bpt^i > Bpt^z = Bpt^e = Bpt^z = Bpt^r \). Histological sections were made of the heads of individual flies with the \( Bpt^e \) allele that do not show laminar peaks. In these sections the lamina appears to be compromised. Specifically, they are reduced and are disconnected from the retina. This observation suggests a possible explanation for the absence of laminar function in these mutants, as characterized by their electroretinograms. Given the uncertainties that are known to be associated with fixation and sectioning, a detailed study will be necessary to determine whether the absence of the laminar peaks is always associated with the disconnection of the lamina from the retina.

**Measuring electrical activity from pupae**

Electroretinograms were measured from 14 \( y w sn \) pupae. Of the 14 pupae, 13 were between 70 to 85 hours post pupariation. One was in advanced stage of development and had partially eclosed from the pupal case. The fly that was partially eclosed showed electroretinograms. The on-transient of the lamina was present while the off-transient was absent. The amplitude was very much reduced compared to the standard ERG. To ensure that the ERG was only in response to light and not an artifact of any stimulus caused by the cameral shutter, the experiment was repeated. The lamp was turned on and light stimulus was provided to the fly only by blocking and removing the light using a piece of black paper. Figure 15 shows the on-transient in the ERG. Electroretinograms could be consistently measured. Electroretinograms could not be recorded from the younger pupae. The electrical circuit was maintained but there was no response to the light stimulus, Figure 16. Unpublished results from T. E. Hanson support our evidence that \( Drosophila \) pupa do not have electroretinograms. This situation is unlike \( Musca \) where visual function can be assayed in the form of ERGs in the pupae.

**Timing and location of cell death**

Previous work had shown that individuals with a \( fa^e; Bpt \) genotype have abnormal
Figure 15. ERG of y w sn pupae shows on-transient. The ERG was recorded at 50mV/D and a speed of 0.2sec/D. The stimulus lasted for 1sec and is shown as a bar.

Figure 16. ERGs of younger pupae do not show any response to the light stimulus which is seen as a white bar.
patches of dead cells in the eyes. Histological sectioning and staining with anti-HRP antibodies had indicated that the optic lobe receives innervation from the retina indicating normal development and then retinal and laminar cells in the patch region die (Duus et al 1992). In order to further investigate the spatial and temporal events of this cell death, we stained whole mounts of retina and lamina with trypan blue. Trypan blue is an exclusion dye which enters dead cells and cannot be transported.

The lamina showed extraordinary amounts of cell death as indicated by trypan blue when stained before the appearance of visible degeneration in Figure 17, A and B. An intense patch of blue appears in the lamina in the same location as the future black patch. This can be visualized in two ways. The retina is transparent and the blue stain is visible through the retina and is seen as a patch underneath the retina. The region of the blue patch is the region of the lamina. At the resolution we have used in our experiments we are unable to tell if this patch of blue stain is in the lamina or in the interconnections of the retina and lamina. Henceforth we will refer to the stain as being in the lamina. The retina covers the lamina like the flat top of a mushroom covering the top of the stem. Pulling back the retina is the second method of visualization. This exposes the tissue underneath the retina. An arc of trypan blue staining is seen in the region apposed to the retina. This clearly indicates trypan blue staining in the region of the lamina. The results presented above indicate that cell death produced by Bpt mutations can be detected using trypan blue staining. Cell death in the lamina of the mutants reduces the tissue integrity. Manipulation of the mutant tissues was much more difficult than dissecting the retina and optic lobes from the control genotypes. In the Blackpatch mutants, when the surrounding tissue which holds the visual system in place was removed, the lamina frequently crumbled and disintegrated. The control experiments which included trypan blue staining of whole mounts of w^fa^ did not show blue stain in the region of the lamina shown in Figure 18.

Blackpatch mutants have a black patch of melanized tissue on the retina at eclosion.
Figure 17. Trypan blue staining of the optic lobe of $w^{fa}; Bpt^t$ pupae at 50 hours. The lamina shows blue stain indicating cell death in two complexes, (A) and (B). R indicates retina and L indicates lamina.

Figure 18. Trypan blue staining of the optic lobe of control pupae. The lamina does not show cell death. R indicates retina and L indicates lamina.
Previous work had shown that this patch of dead cells is visible in the pupae 72 hours after pupariation. Trypan blue staining of \textit{Blackpatch} mutant retina shows a patch of dead cells corresponding in location to the future black patch when the retina is stained before the time at which degeneration becomes visible. The arrow in Figure 19 points to this region in the retina. Just as in the lamina, the tissue integrity is greatly reduced and the retinal cells in the region of the patch are prone to exploding at the slightest pressure. Figure 20 shows trypan blue staining of a \textit{yw sn} retina and this does not show a patch of blue indicating dead cells.

We next used trypan blue staining to determine the precise time in development at which cell death first appears in the retina and the lamina. The results are presented in Table 11 and 12. The time of death was determined by dissecting and staining pupae collected as white prepupae and held for a carefully timed interval before dissection. Cell death is first seen in the lamina. 22\% of the pupae show trypan blue staining in the lamina.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Age of pupae (hours pp.)} & \textbf{Total incubated with stain} & \textbf{Total scored} & \textbf{\% of pupae showing stain in the lamina} \\
\hline
36 & 54 & 32 & 22 \\
40 & 36 & 31 & 35 \\
42 & 48 & 43 & 84 \\
44 & 127 & 118 & 75 \\
46 & 140 & 138 & 72 \\
48 & 92 & 81 & 80 \\
50 & 19 & 18 & 89 \\
\hline
\end{tabular}
\caption{Trypan blue staining of retina - lamina whole mounts dissected from \textit{w^{fa}; Bpt} pupae.}
\end{table}
Figure 19. Trypan blue staining of the optic lobe of \textit{w^{fa}}: \textit{Bpt}' pupae. The arrow points to the region on the retina (R) that shows staining indicating the presence of dead cells in the region that will become part of the future melanized patch.

Figure 20. Trypan blue staining of the optic lobe of control pupae. The arrow points to the retina, R that does not show stain indicating the absence of dead cells in the region.
at 36 hours post-pupariation (pp). At 40 hours pp., 35% of the pupae show cell death in the lamina. There is a sharp peak of death at 42 hours, shown by 84% of the pupae. The cell death stays steadily high at 75%, 72%, 80% and 89% at 44, 46, 48 and 50 hours after pupariation. In these pupae, only the lamina shows cell death by trypan blue staining. The retina looks completely normal and does not stain with trypan blue at these times. There is a regular progression in the pattern of laminar staining. In the younger pupae, the trypan blue stain is seen only as a few spots. Pupae stained at 44, 46 and 48 hours show steadily increasing amounts of staining in the lamina. Pupae older than 48 hours show a decrease in staining and by 54 hours trypan blue stain in the lamina is seen as very fine spots. The wild type pupae and w^fa^ pupae, which are the controls, do not show significant staining in the lamina at any times.

Trypan blue staining of retina and lamina complexes at later times is shown in Table 12. While the younger complexes never showed stain in the retina, the older complexes starting at 56 hours pp showed a patch of blue stain in the retina. The number of complexes showing stain in the retina increases with the age of the pupae. The sample sizes of the complexes of older pupae is smaller because the tissue is extremely fragile and difficult to dissect. The region of trypan blue staining in the retina corresponds to the future region of the black patch. Unlike the laminar stain, the trypan blue stain in the retina appears throughout the entire region of the patch at the same time. The staining region does not appear as a small spot and then increase in size (area covered) with age as in the lamina. Instead, the entire region corresponding to the patch stains blue indicating dead cells. Trypan blue stains the patch region of the retina well into 72 hours when the physical degeneration begins. Trypan blue stained dead cells adjacent to melanized dead cells were frequently seen in the older pupae. To summarize, cell death is seen both in the lamina and the retina. Laminar cell death begins 16 to 20 hours before cell death begins in the retina, reaches its peak and then declines before retinal staining is apparent.
Table 12. Older pupae show trypan blue stain in the retina.

<table>
<thead>
<tr>
<th>Age of pupae (hours pp)</th>
<th>Total scored</th>
<th>% showing stain in retina</th>
</tr>
</thead>
<tbody>
<tr>
<td>54</td>
<td>29</td>
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<td>64</td>
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<td>14</td>
<td>21</td>
</tr>
<tr>
<td>68</td>
<td>8</td>
<td>25</td>
</tr>
</tbody>
</table>

Nature of cell death in the lamina and retina

The TUNEL technique was used to investigate the nature of cell death in the retina and lamina. The TUNEL technique helps to identify the DNA which is fragmented in dying cells, a characteristic of apoptotic cell death. Apoptosis is an active form of cell death, induced by the action of the genes involved in the programmed cell death pathway. We identified intense stain due to TUNEL in the region of the lamina. This was very obvious in the retina - lamina complexes from 46 hr. pupae (Fig. 21, A). This indicates that the cell death seen in the lamina is apoptotic cell death. TUNEL staining of control complexes of the w^{fa^e} genotype do not show any stain in the lamina at these times (Fig. 21, B). TUNEL staining of both mutant, w^{fa^e}:Bpt' and control, Ore R do not show apoptotic cell death in the retina (Figures 22, A and B).

The ocelli of w^{fa^e}: Bpt' flies are normal

_Drosophila_ have 3 ocelli or simple eyes located in a triangle on the vertex of the
Figure 21. (A) TUNEL staining of the whole mount of retina and lamina complex of a $w^{fae}: Bpt'$ pupa, 48 hours after pupariation. The dark spots indicate apoptotic cell death in the region of the lamina. (B) TUNEL staining of optic lobe complexes of the control genotype. There is no indication of apoptotic death in the lamina of the control.

Figure 22. TUNEL staining of the retina of $w^{fae}: Bpt'$ (A) and staining of the retina of control genotype (B). The retinalae do not show the presence of apoptotic cell death.
head. They are considered simple eyes because unlike the compound eyes, they do not consist of several repeating units. Each ocellus contains a thick planoconvex lens over a layer of cuboidal corneagenous cells (Stark and Sapp, 1989). Underneath this, each ocellus has 80 retinula cells that form rhabdoms at their upper ends. The axons from the retinula cells project to ganglion cells. From each ganglion, 4 interneurons project to the brain. There exist three types of ocellar mutations, a) mutations that are specific to the ocelli, for ex., oc mutants have no ocelli, b) mutations that affect the compound eye and the ocelli, for ex., norp A mutants lose their ocellar rhabdomeres as they grow older, and c) mutations that affect only the compound eye. There is some evidence that in rdg A mutants some ocellar receptors begin to show signs of degeneration after one week.

To identify whether Bpt affects the ocelli, flies of the w*fa^x; Bpt^ genotype were examined carefully under the dissecting scope for any abnormalities in ocellar structure. No abnormalities were found with the ocelli. There was no degeneration of the ocelli. Even the flies with the biggest black patches in the compound eyes never showed any degeneration in the ocelli. We can conclude that the black patch due to Blackpatch is unique to the compound eye and underlying optic lobes.

Observation of the deep pseudopupil

The compound eye, when illuminated from below, shows seven spots of light in the same arrangement as the rhabdomeres in an ommatidium. The rhabdomeres have a trapezoid arrangement with six photoreceptors bordering the trapezoid with the seventh one in the middle. Since the eight photoreceptor is underneath the seventh photoreceptor, there are essentially seven columns through which light passes. The innervation from six axons of the ommatidium reach six cartridges while the seventh one passes through to the medulla. Thus there is a superimposition of the trapezoidal structure on the lamina as well. The light comes through the laminar and retinal columns and is seen as seven spots of light. This is called as the deep pseudopupil. Since the presence of the normal pseudopupil is dependent
on the correct structure of the retina, and its innervation of the lamina, any abnormalities in
the pseudopupil will indicate differences in the same.

Flies of the control genotype \( w^{fa} \) were examined for the pseudopupil. The
pseudopupil is not obvious in these mutants because the \textit{facet-glossy} mutation disrupt the
orderly structure of the retina. This means that it would be doubly difficult to score the
deep pseudopupil in individuals with a \( w^{fa} : Bpt \) genotype, the patch also interferes with
retinal and laminar structure. To discover if the \( Bpt \) mutation affects the pseudopupil, flies
with the genotype \( +; Bpt^t \) were constructed (as explained in Materials and Methods) and
observed. These flies have a normal pseudopupil. This indicates that the structure of the
retina and its innervation of the lamina is normal.

\textbf{Discussion}

A primary goal of these experiments was to determine the details of the \( Bpt \) mutant
phenotype so as to test our two hypotheses about the function of \textit{Blackpatch}. If \( Bpt \)
normally functions in the visual transduction pathway, and \( Bpt \) mutations kill neurons in
the eye and optic lobe by blocking or altering the function of this pathway, then the \( Bpt \)
mutant phenotype should resemble mutations in genes of the visual transduction pathway,
such as the \textit{rdg} mutations. Alternatively, if \( Bpt \) normally functions in a neurotrophic
pathway, and \( Bpt \) mutations kill cells by blocking or altering the function of this pathway,
then the \( Bpt \) mutant phenotype should resemble the phenotypes produced by mutations that
inactivate or alter neurotrophic factors. No such mutations are known in \textit{Drosophila}, but
neurotrophic mutations are known in the mouse and in avian systems, and the effect of the
loss of a neurotrophic factor in these systems has been well documented (Bothwell, 1995).

\textit{Blackpatch does not function in the visual transduction pathway.}
Three lines of evidence suggest that Bpt does not function in the visual transduction pathway. The first and most important concerns the function of the visual system in Bpt mutant flies. Our measurements of electroretinograms indicate that the retinal and laminar cells in flies with a w^fa^; Bpt^1 genotype process light and have a normal ERG. Cells in the patch region, being dead, clearly do not respond to light but the surrounding, surviving cells process light normally. The fact that these cells are mutant for Bpt does not affect their electrical activity in response to light. The presence of small, medium, or even large patches of cell death does not affect the ability of surviving cells to respond to light. The amplitude of the ERG does decrease as the patch size increases. However, this reduction in amplitude does not affect the shape of the ERG, and both the retinal and laminar components of the ERG are present. As the patch size increases, there is less of the visual system available for function. Since the ERG is an average of the total electrical activity in the visual system, the reduction in the number of surviving ommatidia reduces the total electrical activity and therefore the amplitude of the ERG. The normal shape of the ERG even in cases of extreme reduction in amplitude clearly illustrates that those cells that survive in the visual system of Bpt mutants function normally. Mutations of the genes that function in the visual transduction pathway do affect the ability of the cells to respond to light (Johnson and Pak, 1986). For example, the rdg mutations affect the visual transduction pathway and hence, the electrical activity as measured by ERG is abnormal, even in eyes that appear normal. Thus Bpt mutations differ from rdg mutations in the most fundamental aspect, they do not alter the function of the visual transduction system.

The second line of evidence is the pattern and timing of cell death. The induction of cell death in the eye by Bpt mutations does not show certain characteristics that are standard for rdg mutations. In individuals with rdg mutations the photoreceptor cells degenerate only after exposure to light, this degeneration is randomly distributed throughout the eye, and it is progressive. In rdg mutants the number of dead retinal cells increases as the fly
ages. Typically an \textit{rdg} mutant fly will eclose from the pupal case with no visible sign of eye degeneration and degeneration will only begin after exposure to light (Johnson, Frayer and Stark, 1982). This pattern of cell death is expected as it is the abnormal processing of light stimulus that kills cells in the \textit{rdg} mutant ommatidia. Our analyses and previous studies have shown that the \textit{Bpt}-induced cell death occurs before eclosion and before the visual transduction system begins to function. Flies with \textit{Bpt} mutations do not show progressive degeneration, nor is the death dependent on exposure to light, as it occurs in flies that have been reared entirely in the dark. The size of the patch remains constant after eclosion (Duus et al. 1992). A final characteristic is the effect of \textit{rdg} mutations on the ocelli. The \textit{rdg A} gene codes for a cyclophilin and is part of the visual signal transduction pathway (Shieh, 1989). Mutations of this gene also affect the ocellar photoreceptors, causing a degeneration or loss of ocelli. \textit{Bpt} mutants do not have altered or missing ocelli.

\textit{The focus of the Bpt mutation is the lamina}

The third line of evidence is the focus of the \textit{Bpt} mutation. The focus of a mutation is the tissue in which the normal function of the gene is needed. A mutant's focus is usually defined in \textit{Drosophila} in somatic mosaic experiments. Classically, the focus is the tissue which must be mutant in a somatic mosaic for the individual to show a mutant phenotype. A mutant focus is sometimes considered to be the tissue which first shows signs of abnormal development or cell death in mutant individuals. Mutations of genes that function in the visual transduction system have their mutant focus in the retina, centered in the photoreceptor cells. \textit{Bpt} mutations have their focus in the lamina. Our developmental analysis of the \textit{Bpt}-induced cell death indicates that the degeneration caused by \textit{Bpt} involves both the retina and the lamina. Degeneration is seen only after innervation of the lamina by the retina takes place, and cell death is first seen in the lamina. In a somatic mosaic analysis, individuals with a mutant retina and a normal optic lobe were generated, and these individuals did not develop a patch in the retina (Duus, Welshons and Girton,
91

In an imaginal disc transplantation analyses where Bpt eye-antennal discs were transplanted into the abdomens of third instar larvae, the retina did not develop a patch, even when the host was a Bpt larva and the host eye did develop a patch (Duus et al. 1992). These results indicate that the focus of Bpt is not in the retina, and suggest that it is instead in the lamina.

Our electroretinogram analysis did show one additional interesting result. The laminar peaks are absent from the ERGs from some of the flies with certain Bpt alleles. The alleles Bpt' and Bpt\textsuperscript{2} always have normal electroretinograms with both retinal and laminar activity. Of the other alleles, Bpt\textsuperscript{2}, Bpt', Bpt\textsuperscript{4} and Bpt\textsuperscript{6} never show laminar peaks. Among flies with Bpt', Bpt\textsuperscript{5}, Bpt\textsuperscript{4} and Bpt' alleles, a variable percentage of flies show laminar peaks. The important question is why these peaks should be absent. The fact that certain alleles should show variability for this phenotype is not surprising. Given the variability in expression and penetrance of the retinal cell death patch shown by Bpt alleles, it is not unusual that this laminar effect should also be variable. However, it is interesting that the individual flies that do not give a laminar peak never have a retinal death patch. Attempts at staining the visual system complex of flies with the Bpt\textsuperscript{4} allele were not successful since the lamina had no integrity and fell apart really easily. This suggests that this laminar ERG effect is associated with a strong Bpt effect on the lamina. In a further analysis, heads of individuals with no laminar peak were fixed, sectioned, and stained to show neural cell structure. In these sections the lamina appeared greatly reduced and disconnected from the retina (data not shown). These results are only preliminary, as the number of heads sectioned was small, however they suggest an important point. The laminar death can be separated from the retinal death. Apparently Bpt-induced death and degeneration in the lamina can occur without a corresponding patch of retinal death. This separation appears to occur when the lamina is showing extremely strong Bpt effects. Considering the other evidence that the Bpt mutations affect the lamina, we conclude that
the absence of laminar peaks in some flies may be due to a strong effect of \textit{Bpt} on the gross laminar structure, and possibly to the induction of an overwhelming amount of cell death in the lamina that either leaves the lamina nonfunctional or disconnects it from the retina in a way that does not induce retinal cell death.

\textit{Bpt} may function in a neurotrophic pathway

Our second hypothesis is that \textit{Blackpatch} may function in a neurotrophic pathway in the development of the eye and/or optic lobe. In mammalian systems, developing neural systems require a wide variety of neurotrophic factors (Korsching, 1993). Where they have been identified, neurotrophic genes show three fundamental characteristics: 1) Each neurotrophic factor is required by specific sets of neurons at specific stages in development, 2) Neurotrophic function requires cell-cell communication, and 3) Neurotrophins function to repress the activation of the programmed cell death pathway, and their loss or inactivation leads to the programmed cell death of the target cell. NGF or nerve growth factor is necessary for the maintenance of sympathetic ganglion neurons and for the survival of certain subpopulations of sensory neurons in the dorsal root ganglia and the trigeminal ganglia (Snider, 1994). Cells of the peripheral nervous system do not require NGF but express two other types of neurotrophins, BDNF and neurotrophin-3 (NT-3). Experiments with knockouts of NGF in mice have shown different time windows for NGF dependence. The dorsal root ganglion cells have an early requirement for NGF while the sympathetic ganglia have a later requirement. The stages of requirement are identified as the lethal period of the mutations. All the neurotrophins known so far function to prevent neuronal programmed cell death (Bothwell, 1995).

Neurotrophic factors or gene pathways have not been previously proven to exist in \textit{Drosophila}. \textit{Dtrk}, a \textit{Drosophila} gene has been cloned and found to be highly homologous to the \textit{trk} family of neurotrophin receptors. The gene encodes a neural cell adhesion molecule and is expressed in the embryonic nervous system (Pulido, 1992). In reference
to optic lobe development, there seems to exist an anticipation or expectation that neurotrophic factors exist. "Some of the phenomena uncovered in these studies (the morphogenetic dependence of columnar cells on centripetal innervation, target-dependent cell death and the developmental plasticity seen among optic lobe neurons following perturbations to their normal afferent innervation) would in a vertebrate system suggest the action of a growth factor, or factors" (Meinertzhagen and Hanson, 1993). We propose that Bpt may function in a neurotrophic-like pathway. If this hypothesis is correct, then Bpt mutations block or alter the neurotrophic pathway, and the Bpt-induced cell death results from the absence or alteration of a neurotrophic factor. Bpt -induced cell death should then show the same three characteristics described previously. To test this hypothesis we made a detailed study of the temporal and spatial localization of Bpt -induced cell death and examined the type of cell death. To understand this study it will be useful to review some specific events of normal neural development in the Drosophila adult visual system.

The undifferentiated eye disc begins to develop into a retina during the third larval instar (Wolff and Ready, 1993). The photoreceptors in the retina send their axons through the optic stalk to innervate the underlying lamina. The outgrowth of photoreceptor axons to the lamina starts with the posterior photoreceptor cells and progresses to the anterior photoreceptor cells. Laminar innervation starts midway through the third instar and continues until 24 hours post pupariation (Meinertzhagen and Hanson, 1993). Growth cone interactions among the photoreceptor axons of the R1 to R6 cells commence during very late in the third instar and continue until 22 or 24 hours post pupariation. The photoreceptor axons reach their laminar targets between 24 to 48 hours post pupariation. The laminar terminal completes development around 48 hours post pupariation. This completion involves the final patterning of the laminar cartridges with the correct numbers of cells in their right locations. Synaptogenesis in the lamina takes place in the second half of pupal development, i.e. between 48 hours to 96 hours.
Programmed cell death is a necessary process in the development of the lamina and retina (Wolff and Ready, 1993; Meinertzhagen and Hanson, 1993). It has a specific pattern, timing, sequence, and function. Cell death in the lamina starts at pupariation, which is approximately 15 to 20 hours after innervation. It is first seen in the posterior region, the region that first received innervations from the retina. It proceeds in a posterior to anterior direction, 3 to 5 cartridge rows at a time. Cell death reaches the anterior edge between 34 hours to 43 hours post pupariation. Cell death in optic lobe development takes place after innervation from receptor axons, but before synaptogenesis, and functions to remove excess cells, cells that do become determined to form one of the normal elements in a cartridge.

The cell death induced by $Bpt$ mutations is characterized by temporal and spatial specificity. In double mutants with a $fa^4; Bpt^1$ genotype, cell death begins first in the lamina, appearing as early as 36 hours post pupariation. As indicated by trypan blue staining, there is an increase in the number of pupae showing cell death in the lamina at 42 hours post pupariation. It stays at a high level throughout 44 to 52 hours. The $Bpt$-induced laminar cell death is seen by trypan blue staining during a) the period (24 to 48 hours post pupariation) when the axons from the retinal photoreceptor cells are reaching their laminar targets, and b) during or after, the end of the period (0 to 34 - 43 hours after pupariation) when normal programmed cell death occurs in the lamina. $Bpt$-induced cell death begins in the retina at 60 hours after pupariation, well after the laminar cell death. Just as there is no increase in the black patch after it is formed, so also there is no progressive death in the retina. Trypan blue staining appears in a large area of the retina at once. Different alleles and even different strains of $Bpt$ with the same allele show characteristic sizes of retinal patches. Siblings of the flies that were dissected and stained were allowed to develop and their retinal patches were examined. The size and location of the trypan blue staining and the final patches were comparable. There were no pupae with
intermediate size patches, indicating that the entire region of the retina that forms the patch begins to die at the same time.

The TUNEL technique is widely used to determine whether cell death is apoptotic (programmed cell death) or necrotic. By modifying the standard TUNEL technique we were able to use this technique on the retina-lamina complexes to determine the nature of the Bpt-induced cell death. Significant staining occurred in the lamina during the time of Bpt-induced cell death. This result indicates that the Bpt-induced cell death is programmed cell death. Thus Bpt-induced cell death exhibits several of the standard characteristics of neurotrophic-induced cell death; limitation to a specific population of neurons, spatial and temporal specificity, and programmed cell death.

The final characteristic of neurotrophic factors is that they require cell-cell signaling pathways for normal action (Korschning, 1993 and Bothwell, 1995). This may explain one of the most puzzling characteristics of Bpt-induced cell death, the requirement for facet. The facet mutations are recessive alleles of Notch, and Notch is active in cell-cell signaling in many tissues during several developmental stages in Drosophila (Artavanis-Tsakonas, 1988; Markopoulou, Welshons and Artavanis-Tsakonas, 1989). A well-documented function of Notch is to send lateral inhibitory signals to adjacent cells. These signals inhibit neighboring cells to adopt the same cell fate as the cell expressing and using Notch. Mutations at this locus interfere with this signaling mechanism and cause over-production of one cell type and a deficiency of the other. For example, embryos homozygous for amorphic Notch alleles have an over proliferation of neuroblasts and consequent hypertrophy of the nervous system (Artavanis-Tsakonas, Delidakis and Fehon, 1991). Individuals homozygous for the recessive facet alleles have an over proliferation of secondary pigment cells and a reduction in primary pigment cells (Cagan and Ready, 1989). There is also a decrease in the normal numbers of undetermined cells and of programmed cell death. In facet mutants, the decrease in programmed cell death is due to the
adoption of the secondary pigment cell fate by the cells that normally would have remained undetermined and would have died. The failure of Notch “protects” these cells by allowing them to adopt an acceptable cell fate and escape programmed cell death. The temperature sensitive allele of Notch gives the same eye phenotype as facet-glossy when the mutant flies are raised at a restrictive temperature (Shellenbarger and Mohler, 1975). This suggests that the facet alleles cause a tissue-specific loss of function of Notch. The fact that Bpt-induced cell death only occurs in individuals who are also expressing a facet phenotype indicates that Bpt interacts with Notch in the eye and/or optic lobe. The cell death phenotype may represent a synergistic interaction, and synergistic interactions are usually taken as an indication that the two interacting genes function in the same pathway or process. Thus Bpt requires the reduction or blockage of a specific cell-cell communication pathway to induce cell death in the eye.

The role of Notch in the developing lamina is not well understood. There is the prediction of a maintenance function for Notch in the adult optic lobe (Markopoulou and Artavanis-Tsakonas, 1991). Since the interaction of Bpt with the temperature sensitive allele of Notch gives a similar phenotype to the fa*; Bpt double mutants, we suggest that the effect on the lamina is similar to the retina, a tissue-specific loss of Notch function (Girton, unpublished results). We can expect that this causes a loss of lateral communication, which may result in an over proliferation of a certain cell type and the reduction of another. The facet mutation alone does not disrupt the structure of the laminar cartridges very greatly or destroy its function. This is supported by the evidence that facet flies do not show trypan blue staining in the lamina and they have normal electroretinograms. It is possible that the mutant phenotype of a facet lamina may not be obvious under light microscopy. Detailed descriptions of the ultrastructure of the wild type Drosophila lamina is so recent, Meinertzhagen, 1991, that there is no thorough study of the mutant optic lobe architecture.
The fact that Bpt-induced cell death depends of the facet phenotype strongly implies that the normal Bpt function interacts with the lateral cell-cell communication system.

The nature of this interaction is not clear. A number of hypotheses could be proposed. For example, the eye-specific facet mutation may block a lateral inhibitory signaling pathway in the lamina. This may causes the over abundance of a certain cell type that has a specific requirement for the Bpt neurotrophic pathway to prevent programmed cell death. Bpt mutations that lower the function of the neurotrophic pathway may result in the reduction of the amount of a neurotrophin. This reduction alone is not enough to cause cell death in the laminar cells, so flies with the genotype, \(N^+; Bpt\) do not show cell death in the lamina nor in the retina. In double mutants of \(fa^+\) and Bpt, there is both the increase in a requirement for the function of the Bpt pathway and a simultaneous reduction in the activity of this pathway. This synergistic interaction pushes cells in the posterior region of the lamina, that, being the oldest have the greatest need for the neurotrophic factor, below the threshold for viability, and the programmed cell death pathway is activated.

The results of our studies support the hypothesis that Bpt is an essential part of a neurotrophic pathway that is functioning during the development of the optic lobe. This hypothesis can explain the basic events that occur during the formation of the Bpt eye spot. The time of axonal innervation into the lamina is a time of intense growth in the visual system. It is a time of requirement of growth factors for the development and maintenance of the nerve cells. Since two different tissues are developing and interacting, there may be a complex medley of factors necessary. Previous studies of the development of Bpt mutants have shown, at least under light microscopy, that the retinal axons develop normally at first (Duus, Welshons and Girton, 1992). They reach their laminar targets and stimulate laminar development. If Bpt normally functions in a neurotrophic pathway, then this factor is required by laminar cells during the later part of the innervation/maturation phase, but before synapsis occurs. Our results are consistent with the reduction in Bpt
function in mutant individuals causing a reduction in the amount of a neurotrophic factor, which causes laminar cells to activate the programmed cell death pathway, the normal response of a neuron deprived of an essential neurotrophic factor. This death then may feed back to the retinal photoreceptor cells that have innervated the dying laminar cells, causing them to die as well.

The fact that cell death usually begins in and is limited to posterior cells suggests that anterior cells, because they develop later have a lesser requirement for $Bpt$ function, and are thus able to survive a reduction in levels that kills posterior cells. Since one of the main differences between posterior and anterior cells is the time of innervation/differentiation, it is tempting to speculate that the need for $Bpt$ function is progressive. Perhaps laminar cells require it continuously after innervation, so that cells innervated first require more. In the mutants without laminar activity, we hypothesize that laminar innervation takes place followed by massive death of the lamina. This causes a complete disconnection of the lamina which results in the absence of the laminar component in the electroretinograms. The disconnection also prevents the retina receiving signals from the lamina causing its death. Therefore we do not see black patches in such mutants.

An alternate hypothesis brings the central neuropil, the medulla into center stage. Certain cells in the medulla are target cells for the innervation from the lamina. Similar to the expectation in the lamina, Notch function may be required for the determination of these target cells. Mis-determination of these cells could cause the failure of $Bpt$, a neurotrophic factor to be secreted. In a classic “neurotrophic” scenario, the laminar axons and subsequently, the laminar cells are killed in a target dependent manner.

At this point we are left with a couple of possible scenarios for the function of $Bpt$ in the developing visual system. Further investigations, including cloning the $Bpt$ locus and analysis of the molecular structure of the gene product may provide the information necessary to understand $Bpt$ function and its role in eye development.
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CHAPTER 3. CHARACTERIZATION OF THE RECESSIVE LETHALITY DUE TO THE BLACKPATCH MUTATION

A paper to be submitted to *Genetica*

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Abstract

The recessive lethality due to the *Blackpatch* mutation was characterized by identifying the lethal phase and analysing the morphology of the nervous system. The lethal phase was identified as being between 48 to 72 hours AEL. This corresponds to the second instar phase in larval development. Anti-HRP antibody staining shows that the nervous system is normal at the end of embryogenesis. Unlike the adult visual system phenotype that requires an interaction with *Notch*, the recessive lethality is specific to the *Bpt* mutation. This clearly indicates that *Blackpatch* has a vital function during early development. The lethal phase corresponds to a phase of proliferation of central brain neuroblasts. *Bpt* may be involved in maintenance functions during this period of nervous system development.

*Key words.* larval lethal, larval nervous system staining

Introduction

It is an axiom of genetics that genes whose mutant alleles give a lethal phenotype are genes that have important functions. This is especially true of genes that regulate developmental processes. In *Drosophila*, numerous examples have been discovered of
genes that control segmentation (gap, pair-rule, segment polarity), neurogenesis (Notch), or segment determination (homeotics) that have lethal mutant phenotypes (Nusslein-Volhard, Weischaus and Kluding, 1984; Lehmann, Jimenez, Dietrich and Campos-Ortega, 1983; Duncan and Lewis, 1982). All of these mutations produce lethality by making a major alteration, either in segmentation, in cell determination, or in cell differentiation, that so greatly changes the body that it is not compatible with life. The effects of these mutations are readily apparent in an analysis of the lethal phenotype and the mutant phenotype gives clear indications as to the normal role of the gene. For this reason the analysis of a lethal phenotype given by a developmentally important gene is nearly always informative. Most of the well-studied lethal mutations affect embryonic development and are hypomorph or amorphic. The effect of alleles (usually recessive) is to reduce the functional gene product to extremely low levels or remove it altogether, and this loss is lethal for the embryo. There are numerous reasons why so many mutations should be lethal during embryonic development, an obvious one being that so many essential processes take place during the first few hours of development. However, other mutations are known with larval, pupal, or pharate adult lethal periods (Littleton and Bellen, 1994).

The original Bpt mutation, Bpt\textsuperscript{i} was isolated by its adult visible phenotype, and this allele is homozygous viable (Duus, Welshons and Girton, 1992). Four alleles; Bpt\textsuperscript{i}, Bpt\textsuperscript{r}, Bpt\textsuperscript{r} and Bpt\textsuperscript{r} that are recessive lethal or semilethal were initially found to have a lethal period early in development. These four alleles fail to complement each other and the deficiencies that remove the Bpt region of the chromosome. These results suggest that Bpt has a vital function during early development that may or may not be related to its function in the eye, and that there is only one vital function being affected in all four lethal alleles. We wish to determine the precise time or stage of lethality, the genetic conditions under which an individual dies, and details of the phenotype of the lethal individual that may give indications about the role of the gene. It is not always possible to identify the exact
cause of systemic death in the larval stage. The systemic death may be caused due to gross
developmental defects or due to extraordinary amounts of cell death in certain tissues, or
due to a failure of a vital tissue function that does not give a gross alteration in morphology
before the general degeneration that follows death. Characterizing the adult visual system
phenotype has shown us that Bpt affects nerve cells during the time of innervation between
the retina and underlying optic lobes. Therefore a major question in our analyses is
whether the mutants have a normally developed nervous system or does the recessive
homozygous Bpt mutations cause abnormalities in the nervous system.

*Drosophila* is a holometabolous insect and has four distinct stages in development
(Bodenstein, 1965). The embryonic stage lasts for twenty four hours after egg laying.
During this stage, the embryo develops several structures like the nervous system which is
used later in adult life. The larval period lasts for a full four days and has three distinct
stages in development. They are the first instar, second instar, and third instar. The
embryo hatches from the egg and is referred to as a first instar larva. The first instar larva
grows bigger in size and at the end of 24 hours, "molts" into a second instar larva. Molting
refers to an event when the larva sheds its cuticle and enters the next stage of development.
The second instar larva also lasts for 24 hours, at the end of which it molts into a third
instar larva. The third instar larval stage lasts for 48 hours during which time, the larva
gets much bigger, its spiracles evert and it is ready to enter the pupal stage. The pupal
stage lasts for 96 hours at the end of which the adult ecloses from the pupal case.

The insect nervous system consists of the central nervous system (CNS) and the
peripheral nervous system (PNS) (Bodenstein, 1965; Truman, Taylor, and Awad, 1993).
The CNS consists of a supraoesophageal ganglion attached to the ventral segmental ganglia
through a pair of connectives. The PNS consists of the sensory and motor neurons. The
cell bodies of motor neurons and interneurons are found in the CNS while the cell bodies
of the sensory neurons are located in the periphery. The axons of the PNS motor neurons
innervate the muscles. The PNS of the larva is formed in the embryo and degenerates around the time of pupation. The larval CNS is a fused mass of brain and ventral ganglia having 8000 to 9000 neurons that arise from embryonic neuroblasts. These neuroblasts differentiate from the surrounding ectodermal cells. *Drosophila* has one neurogenic period during embryonic life and the second during larval life. In these periods, neuroblasts undergo cell divisions and growth giving rise to neurons (Truman and Bate, 1988). The first phase of neurogenesis is arrested at the end of embryonic life and is recommenced during late first instar larval life. Different regions of the nervous system have different spatial and temporal patterns of neurogenesis. The different regions of the developing central nervous system are the mushroom bodies, central brain neuroblasts, optic lobes, thoracic neuroblasts, abdominal neuroblasts, and terminal abdominal neuroblasts. The central brain neuroblasts and optic lobe neuroblasts begin dividing 10 hours after hatching to mid pupariation. The thoracic neuroblasts begin neurogenesis 30 hours after hatching and the abdominal and terminal abdominals 45 hours after hatching. The brain undergoes extensive neurogenesis. The mushroom body neuroblasts are dividing at the time of hatching while the central brain neuroblasts begin dividing 12 hours later. The neurogenesis in these regions continues throughout larval life.

In this chapter the analyses of the lethal phenotype is presented. The analyses includes an identification of the lethal phase and a description of the nervous system at various developmental stages.

**Materials and Methods**

**Genetic stocks**

The four recessive lethal alleles are *Bpt¹, Bpt², Bpt⁰* and *Bpt¹*. *Bpt⁰* and *Bpt¹* were analysed further. They are maintained in the *w¹fa*xbackground. Three lines of *Bpt¹* without *w¹fa¹* called A3.2, A4.1 and A4.3 were constructed as follows.
The progeny from this cross are $w^{fa^+}/+; Bpt'/TM6, w^{fa^+}/+; Bpt'/TM2, w^{fa^+}/+$; $TM2/ TM6, w^{fa^+}/+; TM6/ TM6$ (dead)$+/ Y; Bpt'/TM6, +/ Y; Bpt'/ TM2, +/ Y; TM2/ TM6,$ and $+/ Y; TM6/ TM6$ (dead). Of these, virgin flies of the $w^{fa^+}/+; Bpt'/TM6$ genotype were collected and the following cross was set up.

$w^{fa^+}/+; Bpt'/TM6 , Tb, Sb X +/ Y; Bpt'/TM6, Tb, Sb$

Among the progeny of this cross, both females and males of 2 genotypes are present. The males are of the genotypes, $+/ Y; Bpt'/TM6, w^{fa^+}/ Y; Bpt'/TM6$. They are distinguishable since one copy of $w^{fa^+}$ gives the mutant phenotype. Only wild type males with the $+/ Y; Bpt'/TM6$ genotype were collected. Among the females the two genotypes are $w^{fa^+}/+; Bpt'/TM6$ and $+/+; Bpt'/TM6$ having the wild type phenotype. Hence virgin females were collected and single pair matings were set up as shown.

$(w^{fa^+})(+)/+; Bpt'/TM6, Tb, Sb X +/ Y; Bpt'/TM6, Tb, Sb$

If the female parent was $w^{fa^+}/+; Bpt'/TM6$, then half of the males were of the genotype $w^{fa^+}/ Y; Bpt'/TM6$ with the white-apricot, facet-glossy phenotype. Such vials were discarded. Only those vials were scored where a hundred percent of the male progeny were wild type indicating that they had the genotype $+/ Y; Bpt'/TM6$. Among such vials, all the females will also be $+/ +; Bpt'/TM6$. Three such lines of $+/ B pt'$ were made with the facet-glossy crossed out. They are A3.2, A4.1, and A4.3.

The recessive lethal stocks are maintained on balancer chromosomes. Larvae, pupae and adults are short and thick in heterozygous and homozygous condition (Tubby). In addition, Tubby larvae have tortuous tracheal trunks. The adults also have short and stubby bristles (Stubble).

Embryo collection and scoring

Plates with corn meal, molasses and agar were used to collect embryos. An average of hundred females and fifty males were set up in the bottles to collect embryos.
For scoring the hatching rate, the plates were set up with two hundred embryos arranged in ten rows of twenty embryos each. They were placed in the 25°C incubator and scored for hatching after 24 hours. Unhatched embryos were scored for dead embryos and unfertilized eggs. Unfertilized eggs are very white and when dechorionated manually on sticky tape, there is no embryo inside. Dead embryos turn dark brown.

**Genetic crosses to obtain twenty five percent mutant lethal homozygotes**

For \( Bpt^t \), the following cross was used.

\[
+/ +; +/ + \text{ virgin females} \quad X \quad +/ Y; Bpt^t/ TM6, Sb, Tb
\]

Virgin females, and males of the \( +; Bpt^t/+ \) genotype were collected. They were allowed to mature for 3 to 5 days at room temperature. The flies were mated and maintained in bottles with egg collection plates. Embryos were collected at frequent intervals. The embryos were manually picked up using a pair of forceps and placed in fresh egg plates as described above. Embryos and larvae were collected for further experiments. The cross in the second generation is as follows.

\[
+/ +; Bpt^t/+ \quad X \quad +/ Y; Bpt^t/+ \]

Twenty five percent of the progeny are \( +/ + \), wild type for \( Bpt \), fifty percent are \( Bpt^t/+ \), heterozygous for \( Bpt \), and twenty five percent are \( Bpt^t/Bpt^t \), homozygous for \( Bpt \).

For \( w^f a^f \); \( Bpt^t \) the following cross was used

\[
w^f a^f \text{ virgin females} \quad X \quad w^f a^f/ Y; Bpt^t/ TM6, Sb, Tb
\]

Progeny that were \( w^f a^f \); \( Bpt^t/+ \) virgin females and males were collected. They were allowed to mature for 3 to 5 days at room temperature and mated. The progeny of this cross were collected as embryos and larvae for further experiments.

**Collecting and scoring larvae**

The larvae were collected on the egg plates already described. For the initial experiments, several larvae (up to 200) were placed on an egg plate just after hatching. A set of larvae on a plate is called a "batch". These larvae were counted 6 hours later and
transferred to a fresh egg plate. The batch was counted again 6 hours later. In this manner, counts were obtained for the first twenty four hours of larval life, that is 48 hours after egg laying (AEL).

For the next set of experiments approximately one hundred larvae were placed on apple juice plates (24g agar, 250ml apple juice, 25g sugar and 10ml Nipagin for a total of 1L) and scored for survival 12 hours later. A hundred larvae were placed on apple juice plates at 12 hours, 24 hours, 36 hours and 48 hours of development. They were scored for viability/ lethality 12 hours later. The embryo hatches at 24 hours into the first instar larva. The development of the first instar lasts for 24 hours, at the end of which it molts into the second instar larva. Data was collected regarding the number of larvae that survive after 12 hours. The plates were scoured for dead larvae and counted. The dead larvae were identified for first and second instar larvae by size and shape of the anterior end.

*Analysing the nervous system*

Antibody staining of nervous system specific antigen was used to visualise the morphology of the nervous system. Three genotypes, Ore R, +; Bpt * and w*fa*; Bpt ^ were used in these experiments. Ore R is the wild type genotype and is used as a control. Virgin female flies were collected and mated with males. The progeny from these flies were collected over one week only. This ensures that the flies are fresh and reduces the number of unfertilized eggs in the population. For the two mutant genotypes, the genetic crosses to obtain twenty five percent homozygotes was set up. This procedure has been explained in detail above.

Embryos were collected on egg collection plates several times in a day at one and one half hour intervals. The plates were placed in the 25°C incubator and allowed to develop. They were fixed at different time intervals given in hours after egg laying (AEL). The time intervals are 10 hours AEL, 12 hours AEL, 14 hours AEL, 16 hours AEL and 24 hours AEL. During the fixation procedure, embryos of one genotype from one time point
were pooled together into a “batch”. In this manner, 18 batches of embryos and larvae were stained for the nervous system. Egg plates were also collected where flies were allowed to lay eggs overnight.

Three genotypes, Ore R, +; Bpt\(^{-}\) and \(w^{f}a^{l}\); Bpt\(^{l}\) were treated simultaneously since Ore R is the control genotype for the two Bpt alleles. Any abnormalities that may arise due to the procedure should be random and affect the wild type (Ore R) equally. Any abnormality due to the mutation will be specific to the +; Bpt\(^{-}\) and \(w^{f}a^{l}\); Bpt\(^{l}\) genotypes. In a population of specimens from two heterozygous parents, there are seventy five percent wild type for the mutant recessive lethal phenotype. This makes for an internal control within the batches containing the specimens with the mutant genotype.

Anti HRP (Jan and Jan, 1982), the antibody of choice recognises a carbohydrate moiety attached to many neural proteins and was the primary antibody that allows the staining of neural cell membranes. The anti-HRP used in these experiments was commercially prepared and had been raised in rabbit. The treatment with the primary antibody was followed by incubation with the secondary antibody, Goat Anti Rabbit.

The embryos were brushed from the plates, rinsed with water and dechorionated for 3 minutes with 50% Chlorox. After rinsing with distilled water and NTX (0.7% NaCl, 0.03% Tx-100), the embryos were fixed with 4% paraformaldehyde and heptane followed by devitellinizing with heptane and methanol. They were washed 3x with methanol followed by 3 washes with PT (50mls 10x embryo PBS, 450mls d/w, 500ul Brig 35 detergent). The embryos were washed with PT for half an hour in the rotator followed by 20 or 30 minute incubation with PBT (50mls PT, 0.1g goat serum). The goat serum is a protein that will attach to random proteins in the embryo. This reduces the availability of non specific antigens available for our antibody of choice. Hence it helps to reduce non specific binding of the antibody and enhances the signal to noise ratio.
The embryos were incubated with the primary antibody (1:200) for 4 hours at room temperature or 4°C over night. Along with this, a batch of embryos that have been collected overnight were incubated with the secondary antibody (1:400) for preabsorption. This was followed by four washes with PT and then blocking again with PBT. They were incubated with the preabsorbed secondary antibody for 4 hours at room temperature or o/n at 4°C. After washing with PT, the embryos were treated with the color reaction. 10 μl of DAB and 1 μl of hydrogen peroxide per ml of 1x PBS were added and the embryos were allowed to incubate in this solution for 8 minutes.

For mounting the embryos using methyl salicylate, they were treated to a dehydration series by washing twice with 1x PBS, 2x 10 min. 70% EtOH, 2 x 10 min. 95% EtOH, and 3 x 15 min. 100% EtOH. Most of the EtOH is removed (100 μl is remaining) and 300 μl of methyl salicylate was added. The embryos were allowed to settle and fresh methyl salicylate is added. The specimens were mounted between 2 coverslips underneath a third coverslip to provide more space for the embryos.

Antibody staining of the nervous system of late embryos and young larvae

Late embryos and larvae have a cuticle secreted by the epidermis. The development of the cuticle is initiated in stage 16 of the embryo just before hatching into the larva. The cuticle is very tough and difficult to digest with ordinary detergents. This presents great difficulties in permeability and prevents penetration of the antibody. The cuticle must be disrupted for the antibody to gain access to the nervous system. The larva also has more tissue for the antibody to access the nervous system. Two methods were tried to improve penetration of the antibody.

The first method was to dissect the larva. The first instar larva is very small and it is not practical to dissect out the entire nervous system from large numbers of individuals. To ease the penetration of the antibody, the larva was dissected and the posterior end was removed. The anterior end of the larva with the intact nervous system was fixed in 4%
paraformaldehyde. This was used for staining with antibodies. A second procedure was to use a sonication step to disrupt the cuticle. This was achieved by a sonication procedure developed by Nipam H. Patel (1994) to stain for the nervous system in old embryos and very young first instar larvae. This procedure is fairly recent and has been adapted from a protocol to stain brine shrimp naupuli. To our knowledge, nobody has reported a comparative study of a mutant nervous system with the wild type using this technique.

Four genotypes were used in these experiments. Ore R and w"fda" were used as the control genotypes and +; Bpt" and w"fda"; Bpt" were the mutant genotypes. Genetic crosses were set up to obtain twenty five percent homozygous mutant specimens (explained above). Embryos were collected on egg plates for every 2 to 3 hours and allowed to develop for 24 hours in the 25°C incubator. The larvae were washed for 1 min. with 50% Chlorox bleach to remove the yeast. They were transferred to glass tubes and fixed in 2ml PEM-FA [(For PEM, 100mM Pipes, 2mM EGTA, 1mM Magnesium Sulphate, pH 7), for PEM-FA, made fresh every time, 9ml PEM, 1ml 37% formaldehyde)] and 2ml Heptane for 20 min. to 1 hour on the rotator. Larvae were fixed for an additional 5 min. with fresh PEM-FA containing 0.1% Tween-20. The fix was removed and the larvae were washed using 1ml 70% EtOH/ PBS. They were transferred to flat-topped 2.0ml eppendorf tubes with flat bottoms (very important!) and washed several times with 70% EtOH/ PBS followed by 95% EtOH/ PBS and 100% EtOH. The larvae can be stored in methanol at -20°C. The embryos were rehydrated by washing with a methanol/PT series (70%, 95% and 100%). This was followed by 2 washes for 10 min. in PT after which 500ul of PT was added to the tube prior to sonication.

The sonication procedure should ideally disrupt only the cuticle but leave the embryo/ larvae and the internal structures intact. The series of methanol washes to dehydrate and subsequently rehydrate the specimens reduces morphological damage. A probe tip sonicator, Artek Sonic Dismembrator Model 150, Artek Systems Corporation
was used. Since this is different from the sonicator suggested in the protocol, some modifications had to be employed.

The 2.0 ml eppendorf tubes mentioned above are important because the probe tip is cylindrical and when used with a conical tip 1.7 ml eppendorf tube, the tip touches the sides of the tube causing the tube to absorb the sound waves and this inhibits the sonication. The suggested output was setting 1, and the time duration for sonication was 15 seconds. An experiment was conducted to determine optimum conditions for sonication with the Artek Sonic Dismembrator. The time duration of 15 seconds was maintained constant because longer sonication times will increase the number of larvae showing morphological damage (Nipam Patel, 1994). Two types of samples were used. A set of egg plates were allowed to develop to 16 hours AEL and fixed. These batches contain late embryos and a few larvae. Another set of egg plates were allowed to develop to 24 hours AEL. Hatching occurs at 24 hours and so this population consists largely of first instar larvae. Not all of them are very young first instar because of some acceleration in development due to conditions in the incubator. Lower power settings were used for the embryos and a higher range of power settings were used on the larvae. A small sample of specimens was taken out from each batch and carefully examined to record the level of damage. The results are shown in Table 1. For the 16 hours AEL collections, the embryos held their integrity very well and showed good staining. Better results regarding penetration were obtained at a power setting of 20. Reducing the power to 15 lowers the amount of damage, but penetration was not too good. It is possible that the extent of damage may be slightly reduced by sonication for a shorter time period. It is more difficult to decide on optimum conditions for the 24 hours AEL batches. A power setting of 30 seems to be too high giving too much damage. Following sonication, the larvae were washed twice for 5 min. in PT and then for 30 min on the rotator. They were blocked again with PBT for 30 min. on the rotator. The PBT was removed, 100 μl of primary
antibody (1:200) was added to the larvae and allowed to incubate overnight at 4°C. After subsequent washes with PT and blocking with PBT for 30 min., the larvae were allowed to incubate in the secondary antibody (1:500) for 4 hours at room temperature or 4°C, overnight. The larvae were washed again in PT followed by the colour reaction.

**Results**

*The homozygous recessive w^fa^; Bpt^ is lethal.*

A cross was set up between flies that were heterozygous for Bpt maintained over different TM balancer chromosomes (TM2 and TM6). In this cross, the adult progeny were scored. Among the progeny twenty five percent are homozygous for Bpt., and all other combinations have unique mutations. The fly with the double balancer rarely survives, but those that do are distinguishable from the fly that is heterozygous for Bpt and either balancer. The different types of progeny and their genotypes are shown in Table 2.

\[
\begin{align*}
\text{w}^\text{fa}^\text{x} / \text{w}^\text{fa}^\text{y} ; \text{Bpt}^\text{x} / \text{TM2}, \text{Ubx} \times X \quad \text{w}^\text{fa}^\text{x} / Y ; \text{Bpt}^\text{x} / \text{TM6}, \text{Sb}
\end{align*}
\]
Table 2. The results of crossing heterozygous $w^fa; Bpt'$.

<table>
<thead>
<tr>
<th>$w^fa; Bpt'$ / TM6, Sb</th>
<th>TM2, Ubx / TM6, Sb</th>
<th>Bpt' / TM2, Ubx</th>
<th>Bpt' / Bpt'</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>0</td>
<td>63</td>
<td>0</td>
</tr>
</tbody>
</table>

Thirty nine flies were distinguishable because they had the $Sb$ phenotype i.e., they had the TM6 balancer and were heterozygous for $Bpt'$. Sixty three flies had the $Ubx$ phenotype, i.e., they had the TM2 balancer and were heterozygous for $Bpt'$. All the flies had one balancer indicating that no homozygous $Bpt'$ survived. The $Ubx$ marker on the TM2 chromosome causes a slight increase in the size of the halteres and is a difficult marker to score since it has variable penetrance. The TM2 balancer was replaced by TM6 with the Stubble and Tubby markers. The Stubble mutation causes the bristles to be short and stubby and homozygous Stubble mutation is lethal.

To confirm the lethality in this stock, the progeny of 10 pairs of flies in a vial and 50 pairs of flies in a bottle were scored. The results are shown in Table 3. The flies were scored for the presence and absence of the Stubble marker on the Tm6 chromosome. All the progeny flies always had the stubble phenotype. This is due to the presence of the Tm6 $w^fa^+/w^fa; Bpt' / TM6, Sb X w^fa^+/Y; Bpt' / TM6, Sb$

Table 3. The lethality of the homozygotes in the $w^fa^+; Bpt'$ stock was confirmed.

<table>
<thead>
<tr>
<th>Stubble phenotype</th>
<th>Wild type phenotype</th>
</tr>
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<tbody>
<tr>
<td>From vial</td>
<td>36</td>
</tr>
<tr>
<td>From bottle</td>
<td>218</td>
</tr>
</tbody>
</table>
chromosome. Therefore all these flies are heterozygous for Bpt'. There were no flies that
could have been homozygous for Bpt'. The experiment was repeated for w^fa^ and w^fa^; Bpt' stocks. Fifty males and females were crossed and their progeny were scored. The
results are shown in Table 4. The total number of progeny from the w^fa^ cross is almost
twice the number of progeny from the w^fa^; Bpt' cross. Since the homozygous Stubble is
lethal, this shows that the homozygous w^fa^; Bpt' are also inviable. The w^fa^; Bpt' flies
are maintained over the TM6, Stubble balancer and so, the adult flies were scored for the
presence and absence of the stubbled bristles.

Table 4. The lethality of the homozygotes in the w^fa^; Bpt' stock was confirmed.

<table>
<thead>
<tr>
<th>Stubble phenotype</th>
<th>Wild type phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>w^fa^</td>
<td>0</td>
</tr>
<tr>
<td>w^fa^; Bpt'</td>
<td>259</td>
</tr>
</tbody>
</table>

The homozygous recessive w^fa^; Bpt' is lethal before the pupal stage.

Stocks of Bpt' are maintained using the TM6, Sb, Tb balancer. Pupae with the Tb
(Tubby) marker are short and fat and easily identified. Seventy seven larvae of all ages, of
w^fa^; Bpt'/TM6, Sb were transferred to a vial and scored later. Fifty five flies and 51
pupal cases were counted. The adult flies always had the stubble bristles and tubby body.
and the pupae were always of the tubby phenotype. This indicates that all the pupae always
had the TM6 balancer. Homozygous w^fa^; Bpt' pupae without the balancer were not
found. The homozygotes die before they reach the pupal stage.

The homozygous recessive w^fa^; Bpt' embryos survive and hatch.

The embryos of a stock of w^fa^; Bpt' heterozygotes were scored for hatching
where twenty-five percent of the embryos would be homozygous for Bpt'. If Bpt' causes lethality in the embryonic phase, then only seventy-five percent of the embryos should hatch. Eight plates were scored with 200 embryos each. Table 5 gives the average numbers of embryos that hatched. Among the progeny, for both males and females, 25% are w^fa^/(w^fa^) (Y); +/+; 50% are w^fa^/(w^fa^) (Y); Bpt'/+ and 25% are w^fa^/(w^fa^) (Y); Bpt'/Bpt'. Twenty-five percent that are homozygous for Bpt are expected to die and only seventy-five percent are expected to hatch. The results show that greater than ninety percent of the embryos hatched. This is much greater than expected.

\[ w^fa^ ; Bpt'/+ \times w^fa^ ; Bpt'/+ \]

Table 5. This table shows the average hatching rate of w^fa^; Bpt' embryos.

<table>
<thead>
<tr>
<th>Average number of dead</th>
<th>Average number of hatched</th>
<th>% Hatched</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.25</td>
<td>164.5</td>
<td>92</td>
</tr>
</tbody>
</table>

To further analyse the embryonic viability of w^fa^; Bpt' embryos, the w^fa^ which was the background genotype was crossed out. In the characterization of the adult visual system phenotype of Bpt, the facet-glossy (fa^) allele interacts with Bpt and the double mutants have a black patch on the eye. The facet-glossy (fa^) mutation is an allele of Notch. Notch is an important molecule necessary for cell-cell signalling that leads to cell determination. In the experiments described above, the flies had the facet-glossy (fa^) allele. This was crossed out and the effect of the Bpt mutation alone was verified. The homozygous lethality does not require the interaction with Notch.

Three lines of flies with only the Bpt' mutation, A3.2, A4.1, and A4.3 were constructed. Crosses were set up to obtain heterozygous Bpt' progeny. These flies were
crossed to obtain twenty five percent homozygotes for $Bpt'$. Large numbers of embryos were scored for hatching rates. The results are shown in Table 6. Eighty to ninety percent of embryos hatch normally which is greater than the expected seventy five percent. This indicates that the homozygous $Bpt'$ is viable. *Notch* is not required for the viability of the homozygous $Bpt$.

*The homozygous lethal $Bpt$ alleles do not complement $Bpt'$*

Three other alleles of the *Blackpatch* mutation, $Bpt^l$, $Bpt^r$, and $Bpt^s$ show homozygous recessive lethality. These three alleles were tested for complementation with

<table>
<thead>
<tr>
<th>Name of the line</th>
<th>Total no. of embryos</th>
<th>% of hatching</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3.2</td>
<td>800</td>
<td>82</td>
</tr>
<tr>
<td>A4.1</td>
<td>495</td>
<td>91</td>
</tr>
<tr>
<td>A4.3</td>
<td>614</td>
<td>84</td>
</tr>
</tbody>
</table>

$Bpt'$. The following three crosses show that $Bpt^l$ and $Bpt^r$ do not survive over $Bpt'$. Some flies that were not Ubx and had to be $Bpt^s/Bpt'$ were seen. The results are shown in Tables 7, 8, and 9. Based on the results of the complementation test, the $w^sfa^s; Bpt'$ genotype was selected for further analysis along with the $+; Bpt'$ flies. Like the $Bpt'$, the flies with the $Bpt'$ allele develop as embryos and hatch but are dead before the pupal phase. Experiments with the wild type and the 2 alleles were conducted simultaneously.

$w^sfa^s/w^sfa; Bpt' / TM2, Ubx \ X w^sfa^s/Y ; Bpt' / TM6, Sb$
Table 7. This shows that Bpt\(^d\) does not complement Bpt\(^l\).

<table>
<thead>
<tr>
<th>w(^\text{fa})(^x); Bpt(^l) / TM6, Sb</th>
<th>TM2, Ubx / TM6, Sb</th>
<th>Bpt(^l) / TM2, Ubx</th>
<th>Bpt(^d) / Bpt(^l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>0</td>
<td>136</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>w(^\text{fa})(^x)/ w(^\text{fa}) ; Bpt(^l) / TM2, Ubx</th>
<th>X</th>
<th>w(^\text{fa})(^x)/ Y; Bpt(^l) / TM6, Sb</th>
</tr>
</thead>
</table>

Table 8. The table shows that Bpt\(^l\) does not complement Bpt\(^d\). The allele, w\(^\text{fa}\); Bpt\(^l\) was used for further analysis along with the +/Bpt\(^l\).

<table>
<thead>
<tr>
<th>w(^\text{fa})(^x); Bpt(^l) / TM6, Sb</th>
<th>TM2, Ubx / TM6, Sb</th>
<th>Bpt(^l) / TM2, Ubx</th>
<th>Bpt(^d) / Bpt(^l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>47</td>
<td>0</td>
<td>125</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>w(^\text{fa})(^x)/ w(^\text{fa}) ; Bpt(^l) / TM2, Ubx</th>
<th>X</th>
<th>w(^\text{fa})(^x)/ Y; Bpt(^l) / TM6, Sb</th>
</tr>
</thead>
</table>

Table 9. Bpt\(^l\) does show some survivors over Bpt\(^d\).

<table>
<thead>
<tr>
<th>w(^\text{fa})(^x); Bpt(^l) / TM6, Sb</th>
<th>TM2, Ubx / TM6, Sb</th>
<th>Bpt(^l) / TM2, Ubx</th>
<th>Bpt(^l) / Bpt(^l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0</td>
<td>45</td>
<td>14</td>
</tr>
</tbody>
</table>

Bpt homzygotes die in the larval stage.

The mutant tubby larva are short and fat and their mouth parts are not so angular, but instead are flat and square. They also have tortuous tracheal trunks. In this experiment, a population of larvae were picked from egg plates at random. The number of larvae with the tubby phenotype were scored against the wild type larvae.
without the tubby phenotype. The larvae were also classified into first, second, and third instar larvae. Table 10 shows average numbers in each class. The wild type larvae were much fewer in number. These are the $Bpt^+$ homozygotes. They never survive to the third instar stage. Two batches of larvae were counted every 6 hours after egg laying to identify the rate of lethality in the 48 hours AEL. Results are shown in Table 11.

Table 10. This table shows numbers of Tb versus Tb* larvae picked at random for first (I), second (II) and third(III) instar larvae.

<table>
<thead>
<tr>
<th></th>
<th>Tb</th>
<th></th>
<th>Tb*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>194</td>
<td>185</td>
<td>8</td>
</tr>
</tbody>
</table>

46% of larvae

0% of larvae

Table 11. This table shows the number of larvae of genotype, $+/Bpt^+$ counted every 6 hours AEL in Batch 1.

<table>
<thead>
<tr>
<th>Hours AEL</th>
<th>Alive</th>
<th>Dead</th>
<th>Missing</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>166</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>129</td>
<td>5</td>
<td>32</td>
</tr>
<tr>
<td>36</td>
<td>126</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>42</td>
<td>111</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>48</td>
<td>97</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>97</td>
<td>26</td>
<td>43</td>
</tr>
</tbody>
</table>
Table 12 shows the results from Batch 2. Larvae will burrow into the food and all efforts were made to track them. But in both sets of data, 6 hours after hatching is the time when many disappear. At this stage in development, they are so small that they are difficult to see if they are lost among the dark food and they disintegrate quickly if they are dead. Four batches each, of genotypes, Ore R and +/Bpt were scored for lethality. These batches were scored 12 hours apart for 72 hours AEL. In this experiment the total number of larvae were reduced to enable searching the plate thoroughly. The results are shown in Table 13.

<table>
<thead>
<tr>
<th>Hours AEL</th>
<th>Alive</th>
<th>Dead</th>
<th>Missing</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>270</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>236</td>
<td>6</td>
<td>28</td>
</tr>
<tr>
<td>36</td>
<td>204</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>42</td>
<td>186</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>186</td>
<td>33</td>
<td>49</td>
</tr>
</tbody>
</table>

Table 13. Percentage distribution of larval death in the 72 hours AEL

<table>
<thead>
<tr>
<th>Genotype</th>
<th>48 hours AEL</th>
<th>60 hours AEL</th>
<th>72 hours AEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ore R</td>
<td>4%</td>
<td>5%</td>
<td>3%</td>
</tr>
<tr>
<td>+; Bpt'</td>
<td>0.6%</td>
<td>4%</td>
<td>9%</td>
</tr>
</tbody>
</table>
To further analyse this trend, several larvae were scored for lethality at every time point between 36 hours to 84 hours AEL. The data is shown in Table 14. The first column shows the genotype. The heading for the second column shows the age of the larvae in hours AEL when they were placed on the apple juice plates. The heading for the third column shows the age of the larvae when they were scored (12 hours after they were placed on the plates). The numbers in the second column indicate the number of larvae placed initially on the apple juice plates. The numbers in the first half of the third column show the number of larvae that were dead. The second half of the third column shows the numbers of missing larvae. Approximately one hundred larvae were scored at each time point. The dead larvae among +; Bpt' were sorted into first and second instars shown in Table 15. More of the dead larvae looked like second instar. The spiracles are not a reliable marker for the second instar for the larvae that are already dead. Instead, size and shape of the anterior end were used to distinguish the second instar larvae from the first instar which were very small and almost oval in shape. Figure 1 shows the percentage lethality in Ore R and +; Bpt' larvae in the different time periods. Regarding the fat; Bpt' genotype, the mutant homozygotes resemble the +; Bpt' genotype in having a lethal period in the larval phase. They survive the embryonic stage and are definitely dead by the pupal stage. There was no conspicuous difference between the two alleles.

The nervous system of mutant embryos is morphologically normal 10 and 12 hours AEL.

The Bpt mutation causes death and degeneration in the developing visual system of Drosophila. The recessive lethal alleles may affect the developing nervous system. The Drosophila larva is simple in organization and has few systems that can be affected drastically to cause lethality. Since the nervous system is one of the most important developing organ systems it is standard practice to look for the effect of a developmentally important gene on the nervous system.
Figure 1. Percentage of dead larvae within time periods (hours AEL shown in the box on the right) is shown in 4 blocks for the +: Bpt' column. For Ore R, the time period between 36 to 48 hours had 0% death and is not shown in the histogram. The time periods for the histogram are in the same order as listed on the right: read top to bottom.
Table 14. Score of viability of larvae every 12 hours

<table>
<thead>
<tr>
<th>Genotype</th>
<th>36 hours AEL (total number of larvae)</th>
<th>48 hours AEL (%)</th>
<th>60 hours AEL</th>
<th>72 hours AEL</th>
<th>84 hours AEL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dead</td>
<td>Dead +</td>
<td>Missing</td>
<td>Dead</td>
<td>Dead +</td>
</tr>
<tr>
<td>Ore R</td>
<td>83</td>
<td>0</td>
<td>5</td>
<td>246</td>
<td>5</td>
</tr>
<tr>
<td>+; Bpt'</td>
<td>463</td>
<td>2</td>
<td>5</td>
<td>1695</td>
<td>10</td>
</tr>
<tr>
<td>fa'; Bpt'</td>
<td>29</td>
<td>0</td>
<td>3</td>
<td>282</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 15. Distribution of lethality between first (I) and second (II) instar larvae

<table>
<thead>
<tr>
<th>Time period AEL</th>
<th>Total number</th>
<th>I instar</th>
<th>II instar</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 - 48</td>
<td>463</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>48 - 60</td>
<td>1695</td>
<td>73</td>
<td>111</td>
</tr>
<tr>
<td>60 - 72</td>
<td>788</td>
<td>67</td>
<td>51</td>
</tr>
<tr>
<td>72 - 84</td>
<td>203</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

At 10 hours AEL

The embryos are mostly at stage 14 in development. Figure 2 (A) shows the staining of CNS in an Ore R embryo. The central nervous system clearly shows staining though a little faint. The development looks normal with the commissures being separated. The ventral nerve cord is extended from anterior to posterior but is not curved around any more. Among Ore R, 24 embryos were stained of which 18 looked normal for the CNS. There are always some on a slide that did not stain or only showed partial stain due to permeability problems. For +; Bpt', 174 out of 185 (94%) embryos showed normal nervous systems (Fig.2, B). The remaining number did not show stain or were broken and could not be scored. Sometimes, the embryos get squashed and present a top down view. Such embryos were not used for scoring. For w^fa; Bpt', 13 out of 24 embryos showed the nervous system to be without abnormalities (Fig.2, C). The other embryos on this slide did not show abnormalities but were in disrepair and could not be scored.

At 12 hours AEL

The embryos are mostly at stage 16 in development. Dorsal closure has been completed at stage 15. This is a morphological marker to help stage the embryos. The staining with anti-HRP antibody is very distinct at this stage. In Figure 3 (A) the arrow
Figure 2. Anti-HRP antibody staining of the nervous system of (A) Ore R embryos aged 10hrs AEL (20x); (B) shows staining of the CNS in +:Bpr' embryos, aged 10hrs AEL (10x). (C) shows staining in fa°:Bpr' embryos, aged 10hrs AEL (20x). The arrows point to the CNS.
Figure 3. Anti-HRP antibody staining of the nervous system of (A) Ore R embryo aged 12 hrs AEL (20x). (B) shows the nervous system of +: Bpr' embryos, 12 hrs AEL (10x). (C) shows the nervous system of w'fu':Bpr' embryos aged 12 hrs AEL (20x). The arrows point to the CNS.
points to staining of nervous system in Ore R embryo of stage 16. Both the CNS and PNS are well developed. The peripheral nerves do not yet circle all the way around. The ventral nerve cord begins to shorten in this stage to eventually reach 40% egg length at stage 17. 43 out of 53 Ore R larvae stained very well and showed well developed nervous system. Of the +; Bpt/ embryos, 266 out of 317 (84%) stained well and showed a well developed nervous system. An example is seen in Figure 3 (B), shown by the arrow. At this stage, axons leaving the CNS and the cell bodies of the sensory neurons in the periphery are visible. At this stage, major abnormalities in the development of the nervous system would have been detectable. Fourteen embryos did not show staining but this did not seem abnormal as compared to Ore R where 5 out of 53 did not show staining. There was no obvious abnormalities in twenty five percent of the embryos at stages 14 and 16. *The nervous system of late embryos and larvae can be stained by anti - HRP and does not show gross defects.*

Late embryos and larvae have a tough cuticle that is very hard to permeabilise for penetration of antibody. This has been overcome somewhat by the sonication procedure suggested by Nipam Patel (1994). The protocol has been optimised for the monoclonal 22C10 antibody by N. Patel. The procedure was adapted to work with the anti - HRP antibody instead. As explained in Materials and Methods, several different power levels were tried to optimise the conditions. Six batches were tried as explained in the Materials and Methods section. Late embryos of the Ore R genotype were visualized. Figure 4 shows the staining of CNS and PNS in an Ore R embryo after sonication. Stage 16 and stage 17 embryos were present. Penetration of the antibody into the embryos was very good. The important point here is that the sonicated embryos do retain a great level of detail. The well developed central nervous system with peripheral nerves is seen and looks normal. According to Campos-Ortega’s classification, stage 17 is the last stage in development after which the embryos hatch. By stage 17, the nervous system is
Figure 4. Anti-HRP antibody staining of the nervous system of late Ore R emryo after sonication (20x). The long arrows point to the CNS and the short arrows point to the PNS.
condensed to 40% egg length. By observation of stained specimens it was not possible to identify if they were late stage 17 or young first instar larvae.

A different batch where the embryos were allowed to develop in the 25°C incubator for 24 hours were fixed and stained with the antibody. These specimens were of the +;Bpt' mutant genotype. These specimens were clearly older and were first instar larvae. The staining with the antibody after sonication helps to visualise the gross structures of the CNS. However, there were some problems due to variability in the level of staining. This was a consistent problem with all specimens. The sonication procedure is harsh and disrupts the larvae. The level of disruption required to allow penetration of the antibody also affects the morphology of the embryo. The number of specimens that show the stain without variability and retain morphology is very limited. In the largest number of cases, the stain permeates only in the region of disruption. For example, if the larva is disrupted in the anterior end, then the optic lobes stain well but the stain does not reach the peripheral nerves in the posterior end.

Dissection of 1 instar and staining with the antibody.

A total of 1,122 larvae of various ages were dissected by cutting the posterior end to help the permeabilisation of the antibody. Whenever possible, the portions of the gut that came out were removed. The larvae were fixed in 4% paraformaldehyde. Of these, 133 at 48 hours AEL (at the boundary of first/second instar) were treated for staining with antibody. Ninety four larvae were of the w^fa^; Bpt' mutant genotype and 39 were w^fa^ genotype. They were stained with the anti-HRP antibody. The specimens were incubated with the antibody overnight to allow maximum time for penetration. No staining of the nervous system was visible. Some background staining was visible near the regions of the dissected ends. It was very clear that the antibody could not penetrate from the posterior end to stain the nervous system. It is important to add that the larvae cannot be dissected from the anterior end because the nervous system is found closely behind the
mouth parts. In fact the ganglia are described as “supra-oesophageal” and “sub-oesophageal” because they are closely associated with the anterior region of the gut. It is impossible to dissect the larvae from the anterior end and leave the nervous system intact. Trypan blue staining of the nervous system to identify cell death.

The nervous system of approximately 25 to 30 second instar larvae were dissected out and stained with trypan blue. There was no difference between the wild type and the mutant nervous systems. The caveat to this procedure is that the lethal phase is in the second instar. The approach is to be able to identify abnormalities like extra ordinary amounts of cell death before the larva dies. Hence it is the bias of the experimenter to choose living larvae to dissect and stain with trypan blue. Since the mutant larvae die in that phase, the choice of larvae will be heavily biased towards wild types.

Discussion

Developmentally important genes are necessary at specific stages of growth in specific cell types and mutations are frequently lethal. The spatial and temporal specificity of the lethality provides information about the spatial and temporal specificity of gene action. It is for this reason that the fundamental questions addressed in studying a lethal phenotype are a) what is the lethal phase and b) what is the organ system that shows the effect of a mutation just before the lethal phase? Since lethality is systemic death, it is not always possible or realistic to find a unitary cause of death.

The Blackpatch allele, +; Bpr<sup>t</sup> was used for analysis of the lethal phase. The lethal phase is during the second instar stage of larval development. This stage lasts for 24 hours and it was subdivided into 2 phases of 12 hours each. 10% of the larvae died in the 48 to 60 hour period AEL. This is different from the 5% death seen in Ore R. In the second half of the second instar life span, 60 to 72 hours AEL, 15% of the mutant larvae die which is quite different from the 3% wild type death. 25% of the population of larvae die in the
second instar stage. This is very close to the expected 25% lethality for homozygous recessive lethality. Among the dead larvae, most were in the second instar stage. The lethal period of the $Bpt$ mutation is thus between 48 to 72 hours AEL.

In the development of the larva at this time period, it is the nervous system that is undergoing spatial and temporal proliferation. It is possible that the $Bpt$ gene product is required during this process. In Chapter 2 we have suggested that $Bpt$ may act in a neurotrophic pathway that is necessary for visual system development. In the larva, $Bpt$ may be needed for a similar function. While the neuroblasts of mushroom bodies undergo proliferation from the embryo itself, the neuroblasts of the central brain and optic lobes start proliferating 34 hours AEL and continue until mid pupariation (Truman and Bate, 1988). This process may require the action of a neurotrophin in the same way that neural crest cells in mice require NT-3, a neurotrophin acting as a mitogen (Kalcheim, Carmeli and Rosenthal, 1992). Since the proliferation occurs over many hours, $Bpt$ may be needed in a pathway that supports and maintains these cells. So far, the procedure for visualising the nervous system has been extended from embryos to the first instar larvae. There is absolutely no precedence for ever achieving this in first instar larvae whose cuticle is even more difficult to permeabilise.

Our analyses addressed the question whether the embryonic nervous system shows morphological abnormalities prior to hatching. The results clearly show that the nervous system is normal in the mutant embryos prior to hatching. In our study, the specimens we have observed have ranged from 10 hours AEL to a maximum of 30 hours AEL. The requirement for $Bpt$ may not commence until 34 hours AEL. The lethal period begins at 48 hours AEL and peaks between 60 to 72 hours AEL. The timing of antibody staining may be too early to visualize abnormalities in the nervous system.

The presence of defects in a particular system provides a possibility that the gene function may be required in those tissues. However the absence of mophological
abnormalities in the embryos that show lethality in later stages of development is not unusual. In an extensive genetic and phenotypic analysis of the 22F1-2; 23B1-2 chromosomal region for genes required in neural development in Drosophila and to identify mutations in the synaptotagmin gene, Littleton and Bellen (1994) isolated 13 essential genes. Of these, 19 alleles of 22Fd, 7 alleles of 23Ab, and 6 alleles of 23Ad show larval lethality. Eight alleles of 23Af and 2 alleles of 23Ag show late larval/pupal lethality. Of all these genes, only one, 23Ab shows structural defects in the CNS and lacks half the neurons in the PNS. The others do not show defects in the embryonic nervous system or obvious defects in their morphology prior to hatching. This example illustrates the idea that systemic lethality at a certain stage is not always preceded by an obvious or visible defect in a specific organ system.

Staining the larval nervous system after sonication helps us to visualise the overall gross structure of the CNS. If there was abnormality in the CNS at the level seen in the visual system defect, in twenty five percent of the specimens, it would be visible. It must also be noted that when a population of larvae are stained for the nervous system during the lethal period, if it shows abnormalities, it will not be possible to differentiate between cause of death and effect of death. Death in an organism, the size of a Drosophila larva quickly shows degeneration. This degeneration can affect the nervous system and cause it to look abnormal.

Unlike the adult phenotype, the homozygous recessive lethality of Blackpatch does not depend on facet-glossy (Notch). This indicates that the lethal phenotype is specific to the Bpt mutation. In the visual system, the facet-glossy localizes it to the eye. In the visual system we suggested that a synergistic interaction between the 2 mutations cause the cell death in the eye. Further we postulated that fa causes the misdetermination of a cell type that requires Bpt and a simultaneous mutation decreases the level of Bpt causing the large scale cell death. The absence of a Bpt-alone specific phenotype in the visual system
may be because the requirement is not as acute or due to a redundancy in the system. In the larva, a reduction in Bpt alone may cause the death because of a greater requirement for Bpt. Absence of Bpt or its mutant effect on a particular pathway may be too deleterious and is not relieved by any other gene products at that time. In the present available literature, genes involved in the nervous system development of the second instar larva do not appear to be elucidated. The work has concentrated largely on the development of the embryonic nervous system. Undoubtedly this is due to the easier accessibility of the nervous system in the embryos.

In conclusion, we state that Blackpatch has a vital function in the development of Drosophila. The mutant larvae are dying at a time of large amount of nervous system development. We hypothesize that Bpt may be necessary to maintain the nerve cells through this developmental period.

References


CHAPTER 4. APPROACHES FOR ISOLATION OF CANDIDATE DNA FROM THE BLACKPATCH REGION

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Abstract

Two approaches were taken to isolate candidate DNA from the *Blackpatch* region. One approach used pulsed field gel electrophoresis to isolate a YAC containing DNA from the *Bpt* region. The second approach was to isolate *P* element insertion mutations of *Bpt*. We were successful in isolating 19 inserts in the *Bpt* region. An analysis of the mutagenesis screen is provided.

*Key words.* pulsed field gel electrophoresis, *P* element mutagenesis

Introduction

The molecular structure of a gene provides important information regarding its function. Cloning provides the physical piece of DNA that represents the gene and allows the determination of the molecular structure. Cloning involves several steps: a) mapping the gene, b) isolating candidate DNA and c) confirming the identity of the DNA. Our objective is to isolate and characterize the *Blackpatch* region. This will help us to identify the structure and consequent function of the *Bpt* gene at the molecular level. Positional cloning and Transposon Tagging were the two approaches taken to isolate this piece of
DNA. This chapter explains the experimental design, the results, and discusses the pitfalls of the two approaches.

Positional cloning depends upon previous knowledge of the location of the gene on the chromosome. In *Drosophila*, information about the location of genes is given by two measures, a genetic map location and a cytological location. The distance between genes as determined by the frequency of recombination gives a genetic map location. Linkage mapping has shown that Blackpatch (*Bpt*) maps to 66.4 between *Delta*, at 66.2 and *ebony* at 70.0 on the third chromosome (Duus, Welshons and Girton, 1992). The salivary gland chromosomes in *Drosophila* have a unique banding pattern that is used as reference points to assign a physical location for a gene. This provides a cytological location for a gene and as such, *Bpt* is located between the 92A6 and 92B1 bands (Duus et al., 1992). Positional cloning isolates a region of DNA whose identity is based on this physical map location.

A second approach to cloning *Bpt* was by transposon tagging. Transposon tagging involves generating a mutant allele of a gene by inserting a P element, thus 'tagging' the gene with a transposon (Engels, 1994; Craig, 1990). The phenotype provided by the insertion event will identify the presence of the P element within the gene. A library of clones made from such DNA will contain pieces of the P element flanking the gene, in this case, *Bpt*. Since the P element is cloned, it can be used as the first probe to isolate DNA containing both P element and *Bpt*.

**Positional Cloning**

The *Drosophila* genome is approximately 165 Mb (165,000 kb) (Hartl, et al., 1992). The euchromatic portion consists of approximately 110,000 kb. The *Drosophila* genome project has provided several YACs, contigs, and P1 clones, vectors that can carry large portions of DNA (Garza et al., 1989; Siden-Kiamos, 1990; Ajioka, 1991; Kafatos, 1991; Merriam et al., 1991; Smoller, Petrov, and Hartl, 1991). While a phage clone can...
cover about 5 to 15 kb, vectors like YACs can carry an average of 200 kb. Four hundred and ninety-six YAC clones cover the entire euchromatin of the fly. There exist yeast cell lines that contain the specific YAC of interest. The YAC designated NO7.75 contains 450Kb of DNA from the chromosomal region that included *Blackpatch*. It is maintained and propagated in a standard AB1380 strain of yeast. The YAC can be isolated from the yeast cells using Pulsed Field Gel Electrophoresis (Schwartz and Cantor, 1984; Burke, Carle, and Olson, 1987; Silverman, et al., 1989; Kafatos, et al., 1991). The DNA from the YAC is used to construct a sub-genomic library containing sequences from the *Bpt* region. The location of these clones is confirmed by in situ hybridization to polytene chromosomes. A chromosomal walk through the library would isolate the complete region including the *Bpt* gene. In our situation, we could then use ten EMS induced *Bpt* alleles and six x-ray induced revertants to search for the clones containing the *Bpt* gene by mapping the mutant lesions of these alleles.

**Transposon Tagging**

Transposable elements or transposons were originally identified in maize by Barbara McClintock (see Calos and Miller, 1980). They are sections of DNA in a genome, that move and insert in regions where they were not previously present. The insertion event of an element into or adjacent to a gene can induce the mutant phenotype. Its subsequent transposition also introduces many mutations like chromosomal deletions, gene silencing or ectopic expression. Thus, they provide the genome with a dynamic organization. In *Drosophila*, there exists a family of transposons called P elements (Snyder and Doolittle, 1988). They were identified by the phenomenon of hybrid dysgenesis. Hybrid dysgenesis describes the condition in a fly where excessive transposition leads to high rate of mutations that cause sterility. This was noticed in the progeny from crosses of wild type strains with female flies of laboratory strains. The wild type strains had
transposable elements that moved only in the cytoplasmic environment of the laboratory strain. The wild type that has the ability to repress transposition is said to have the P cytotype while the laboratory strains that allow transposition is said to have the M cytotype.

The complete P element has 2907bp with 31bp inverse terminal repeats, transposase binding sites and 4 exons, Exon 0 through Exon 3 that code for the transposase enzyme. This entire structure is the autonomous element (O’Hare and Rubin, 1983; Engels, 1994). The 2-3 intron is a germline specific splice site (Laski, Rio and Rubin, 1986). In somatic cells, this intron is not spliced due to a 97kD protein that binds to a site in exon 2, 12 to 13 bases from the 5' splice site. The 97kD repressor is also present in germline cells at much lesser quantities. The complete element transcribes a 66kD protein that is a repressor of P mobility. On splicing, the exons make the transposase enzyme, an 87kD protein that binds to the transposase binding site of the P element.

P elements have been modified to serve as mutagens and introduce mutations in a gene of interest (Spradling and Rubin, 1982; Engels, 1985; Cooley, Kelley, and Spradling, 1988). The exons that code for the transposase are deleted and replaced by a marker element. When a P element carrying the white gene is introduced into a fly with the white genetic background, the fly has red eyes. This is due to the wild type white protein provided by the normal copy of the gene on the P element. Thus, the presence of the P element is announced by the marker gene. The deletion of the coding region removes a constitutive source of transposase. Transposition occurs only when the modified transposon is provided with a source of transposase. A P element with the following modifications serves as a transposase source. These modifications are, a) deletion of the intron between the second and third exons and b) deletion of one of the terminal repeats. The deletion of the intron allows the P element to code for transposase in both somatic and germline cells and the deletion of the terminal repeat removes its capacity to transpose. One
such element is described as "wings-clipped" and is maintained in a strain of flies that do not have any other kind of P elements (Cooley, Kelley, and Spradling, 1988).

Mutagenesis can be controlled by using single, modified P elements (Cooley, Berg, and Spradling, 1988). Crossing two strains of flies with the two types of P elements provides progeny who carry both the transposon and the transposase source. In these, the transposon responds to the enzyme and moves. Every progeny fly is unique because the P element has transposed to a different location. The initial transposition event is stabilized in their progeny by crossing out the transposase source. Because of the 2-3 intron deletion, the gene encoding transposase is transcribed in somatic and germline cells causing transposition to occur in both. Because of the presence of the white gene on the P element, that transposes at random and lands in regions where it may not be transcribed, the fly with an active P element has mosaic eyes. Such flies are described as "dysgenic".

Euchromatic regions are preferred as insertion sites over heterochromatic regions, though P elements can transpose into heterochromatic regions and function (Berg and Spradling, 1991). There are recognised hot spots in the Drosophila genome. For example, the singed locus has an insertion frequency of $10^{-2}$ while the vestigial locus has a frequency of less than $10^{-6}$ (Hawley, et al., 1988). Prior to experimentation, there is no reason to believe that a gene is more or less accessible to P elements.

Materials and Methods

For Positional Cloning

Preparing the yeast

The YAC was maintained in the AB1380 strain. Yeast cells were grown to stationary phase in a maximum of 50 mls of selective media (-Ura, -Trp), at 30°C. DNA was prepared by lysing cells suspended in solid agarose inserts. The inserts were then
placed into gel slots prior to electrophoresis. A hemocytometer was used to count the number of yeast cells in the solution and measure its density.

The solution of yeast cells is spun down and resuspended in agarose that has been melted in THE (10 mM Tris pH 7.6, 10 mM EDTA, pH 8 i.e., 10 times higher concentrations of EDTA to prevent the action of DNAses). This solution was poured into plastic molds and allowed to solidify for 10 minutes at 4°C. The plugs were incubated in TE with Zymolyase (1 ml solution for 1 plug, Zymolyase = 20 mg/ml in 10 mM Sodium Phosphate pH 7.5) at 37°C for 5 hours. The solution was removed with a pasteur pipette and replaced with ESP solution (0.5 M EDTA pH 9.3, 1% Sarkosyl, 0.5mg/mL Proteinase K) and incubated at 37°C, overnight without shaking. The plugs were washed with THE to remove sarkosyl. The plugs can be stored at 4°C in 0.5M EDTA or THE.

**Pulsed Field Gel Electrophoresis**

The method used was the CHEF. CHEF for Contour-clamped Homogenous Electric Field is the most advanced pulsed field technique available. It provides uniform electric fields. The electrophoresis chamber was an acrylic box with 24 horizontal electrodes arranged in a hexagon. The chamber dimensions was 11 x 43 x 44 cms with a buffer capacity of 2 litres. Replacable platinum wire electrodes were 0.01 inches in diameter. The Pulse wave 760 Switcher alternates the fields, sets the length of the switching intervals and sets the run time. It also permits ramping the switch time from the beginning to the end. Ramping is achieved when the switch interval at the start of the run is different from that at the end of the run. Ramping is very important since it helps to enhance the resolution of fragments in a certain size class of DNAs and reduces the tendency of large DNA fragments to migrate faster than smaller DNAs.

The yeast cells were mixed with agarose and poured into a mold to make the "gel inserts". The gel inserts or plugs containing approximately 1 to 5 ug of DNA were placed in front of the comb and a 1% agarose gel in 0.5x TBE was poured. The voltage was set at
200 volts and it was run for 24 hours at 12°C. The electrophoresis buffer (TBE) was continuously circulated and simultaneously cooled. The pulse parameters were 40 to 50 and switch time was 10 to 14 hours. DNA was visualized with ethidium bromide and photographed using Polaroid 667 film with shortwave UV illumination.

Isolation of DNA

For the initial runs, a molecular 'pulse marker' was used. For subsequent runs, control DNA from the AB1380 strain (including known control provided by Dr. Laura Civardi from Dr. Nikolau's lab, was used). The band representing the YAC was cut out and the DNA was extracted from the agarose slice. After freezing and thawing, the agarose slice was heated to 65°C for 15 minutes in 0.1 vol 3M NaAC and 0.1 vol 1M Tris (pH8). Equilibrated phenol was added and DNA was collected from the aqueous layer.

Details of the YAC

The Drosophila library of YAC's was constructed with the cloning vector, pYACP1. This is modified from the prototype, pYAC2 by the addition of the ends of a P element and the hsp70:g418 resistance gene. Clones designated with N, for ex. NO7.75 were constructed from Not I fragments of Oregon R DNA. The YAC was obtained from Dr. Daniel Hartl's lab, Dept. of Genetics, Washington University School of Medicine, St. Louis. The YAC contains 460Kb of DNA from the region corresponding to the cytological location, 92A6:92C4 (Ajioka, et al., 1991).

Quantitation of the YAC DNA

DNA was quantified by ethidium bromide fluorescent quantitation. A stock solution of DNA (0.55 μg/ul) was diluted to obtain different standards of concentrations 0, 1, 2.5, 5, 10 and 20 μg/ml. 10 μl of each of the solutions was spotted on a piece of saran wrap. 2 μl of Ethidium Bromide was added to them, visualized through a ultra violet light box and photographed. The concentration of DNA was estimated by comparing the intensity of the fluorescence in the sample with those of the standard solutions.
For Transposon Tagging

Genetic stocks

A single modified P element, PGR848 (provided by Dr. Chris Cheney, Washington University) located at 92B1 was used as the transposon. The strong source of transposase, ryPA2-3wc, ry\(^*\) / ryPA2-3wc, ry\(^*\) was used. It is located on 99B and carries the wild type copy of the rosy gene as a marker. All the stocks were maintained in a white-apricot, facet-glossy (w\(wa^a\)) background. They are ryPA2-3wc, ry\(^*\) / ryPA2-3wc, ry\(^*\) . w; PGR848, w\(^*\) / Tm3, Sb, Ser, Df(3R)+1 / Tm3, Sb, Ser (this deficiency extends from 92A15 to 92C2), Df(3R)OF4 / Tm2, Ub5 (this deficiency extends from 92A5 beyond 92C6), cpBpt\(^e\) / Tm2, Ub5 and Df(3R)3003 / Tm6, Tb, Hu (this deficiency extends from beyond 91F11 to 92A8). The deficiencies, +1, 3003 and OF4 have been used to map the region of the Bpt gene. The +1 deficiency flanks the right breakpoint of Bpt, the 3003 deficiency flanks the left breakpoint, and the OF4 deficiency spans the region of Bpt.

The mutagenesis cross

A mutagenesis scheme was designed to obtain a P element insertion mutation in Blackpatch. The screen was extended such that insertion events in the chromosomal region to which Blackpatch is mapped could be identified. This is a 140Kb region between the salivary gland chromosome bands, 92A6 - 8 and 92B1 of the right arm of the third chromosome (Duus et al., 1992). Male flies bearing the P element were crossed with female flies carrying the \(\Delta\) 2-3 chromosome to provide dysgenic hybrid progeny. Male dysgenics were crossed with female flies carrying deficiency chromosomes in three crosses and a fourth cross with \(fa^a; Bpt^f\) flies. Flies carrying this allele are homozygous lethal.

Bill Engels, Welcome Bender and others suggested that female dysgenics should be used instead of male dysgenics since they show higher activity of the P element. Two batches, designated L and M were conducted using female dysgenics. The results from those crosses are described separately. The standard mutagenesis cross is diagrammed in
below. The G0 cross is shown with the dysgenic male. There were certain variations, ex., for batches L and M, G0 dysgenic females were used.

\[ \text{P } w^{fa^z}/w^{fa}, ryP\Delta2-3wc, ry^*/ryP\Delta2-3wc, ry^* X w/Y; GR848w^+/TM3, Sb, Ser} \]

\[ \text{G0 } w^{fa^z}/Y; ryP\Delta2-3wc, ry^*/GR848w^+ X w^{fa^z}/w^{fa}; Df(3R) +1/ TM3, Sb, Ser} \]

\[ \text{G1 } w^{fa^z}/Y; *w^*/Df(3R) +1 X w^{fa^z}/w^{fa}; Df(3R) OF4/ TM2, Ubx} \]

The mutagenised chromosome was tested in the following crosses.

1) \( w^{fa^z}/Y; *w^*/ TM3 \) \( X \) \( w^{fa^z}/w^{fa}; +1/ TM3 \) test for P element mobility

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Phenotypes</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>( w^{fa^z}/w^{fa^z}(Y); ) *w^*/+1</td>
<td>red eyes, normal bristles</td>
<td>P element is active</td>
</tr>
<tr>
<td>( w^{fa^z}/w^{fa^z}(Y); ) *w^*/ TM3, Sb</td>
<td>red eyes, stubble bristles</td>
<td>Stock to maintain mutagenised chromosome</td>
</tr>
<tr>
<td>( w^{fa^z}/w^{fa^z}(Y); ) *w^*/ TM3, Sb</td>
<td>white apricot, facet glossy eyes; stubble bristles discard</td>
<td></td>
</tr>
<tr>
<td>( w^{fa^z}/w^{fa^z}(Y); ) TM3, Sb/ TM3,Sb</td>
<td>white apricot, facet glossy eyes; stubble bristles lethal</td>
<td></td>
</tr>
</tbody>
</table>

2) \( w^{fa^z}/Y; *w^*/TM3, Sb \) \( X \) \( w^{fa^z}/w^{fa}; Df (3003), Dl / TM6, Sb \) locate insert

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Phenotypes</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>( w^{fa^z}/w^{fa^z}(Y); ) *w^*/ Df (3003)</td>
<td>red eyes, delta wings</td>
<td>P element is not in the Df (3003) region</td>
</tr>
<tr>
<td>( w^{fa^z}/w^{fa^z}(Y); ) *w^*/ TM6, Sb</td>
<td>red eyes, stubble bristles</td>
<td>stock for the mutagenised chromosome</td>
</tr>
</tbody>
</table>
The mutagenesis screen was conducted in several batches over a period of one year. Each batch was given a letter designation, A, B,..., S. The batches are separated in time, that is Batch A was set up and screened followed by Batch B and so on. Every bottle within the batch was given a number designation. Each bottle was subcultured three times, that is designated among fly geneticists as bottles, A, B, and C. A colour scheme was set up so that data recorded from the A bottle was in red, from the B bottles in blue and from the C bottles in green. Possible mutants were given the name of the batch and a number. Since they are entered in the notebook with a particular colour designation, a mutant can be tracked back to its parental bottle. The initial batches were smaller consisting of 12 A bottles that were subcultured 3 times resulting in a total of 36 bottles. The later batches
were much larger consisting of 30 bottles, subcultured 3 times resulting in 90 bottles. Each bottle consisted of 17 dysgenic males X 50 +1/TM3,Sb virgin females.

Results

Results for Positional Cloning

Contour-clamped homogenous electric field is a method of pulsed-field gel electrophoresis. It was successfully employed in isolating the YAC, NO7.75, maintained in AB1380 yeast cells. Figure 1A shows an ethidium bromide stained 1% agarose gel with the chromosomes resolved. The AB1380 that does not have the YAC was used as a control. In previous runs, it had been verified that the last 4 chromosomes of the AB1380 have the following sizes, 440 Kb, 350 Kb, 280 Kb and 240 Kb. The expected band size for the YAC is 450 Kb, the size of the YAC, NO7.75. In Figure 1A (top), lanes 1 and 10 are empty of sample. Lane 2 has DNA from AB1380 borrowed from Dr. Laura Civardi. This was the known control DNA. Lanes 3, 4 and 7 have DNA from AB1380. For the DNA in lane 4, the lysis procedure included Proteinase K while for the DNA in lane 7, the lysis procedure included Protease. There is no obvious difference in the appearance of the fragments. Lanes 5, 6, 8 and 9 have DNA from AB1380 that carries the YAC, NO7.75. In lanes 5, 6 and 8 there is an extra band just above the 440 Kb chromosome. This is at the correct region for the NO7.75 with a size of 450 Kb. This band was regarded as the YAC and was cut out. The DNA in lane 9 looks degraded. Figure 1 (B) is the same gel after the correct band has been cut out. The solutions containing YAC DNA from two gels with four inserts each was pooled. The quantity of DNA was measured by ethidium bromide quantitation. The fluorescence in the YAC DNA samples were compared to that of the stock DNA sample. The total quantity of YAC DNA isolated from five gels was 580 nanograms.
Figure 2. The YAC, NO7.75 was successfully isolated using the CHEF system. (A) shows the gel with the 450 kb YAC band seen in lanes 5, 6, 8. A description of the lanes 1 through 10 can be found in the text. (B) The YAC band was cut out of the gel to enable extraction of the DNA.
Transposon Tagging

Table 1 shows the results of scoring the G0 cross of the R batch. A dysgenic male was crossed with +1/TM3 virgin females. The four classes of progeny are *w*/+1 (red eyes, normal bristles), *w*/TM3 (red eyes, stubble bristles), ryPΔ2-3wc,ry*/ TM3 (white-apricot eyes, stubble bristles), and ryPΔ2-3wc,ry*/ +1 (white-apricot eyes, normal bristles). The first two phenotypic classes are relevant and the numbers of flies scored in these classes are presented. The genetic background of these flies is white-apricot that gives an orange colour to the eye. Red eyes indicate the presence of the P element. Control crosses had shown that the +1 deficiency does not complement the P element PGR848, i.e., PGR848/+1 is lethal. The presence of the first class of progeny indicates that the P element has jumped to a new location and in combination with +1 is no longer deleterious. It is a visible proof of P element activity. The cross and progeny are diagrammed in the materials and methods. The siblings from the same transposition event have red eyes due to the P element and stubble bristles due to the Stubble marker on the TM3 balancer chromosome. These flies can be used to set up a stock where the transposed P element, *w+ is maintained. The R mutants, *w*/+1 were crossed with +1/TM3, Sb.

Table 2 shows the partial results of the cross used to test for P element mobility (diagrammed in Materials and Methods). In this cross, our purpose was to identify transposition events where the P element left the third chromosome and jumped to a different chromosome. A fly that has long bristles and not stubbled ones has to carry the +1 chromosome, not the TM3. If the same fly has orange eyes then it does not carry the P element. But it cannot have the +1 chromosome because the homozygous +1 should be lethal. This situation can arise only if the P element has left the third chromosome (-P) that gives -P/+1 genotype. This fly does not receive a copy of the chromosome (for example, one of the II chromosomes) that has the P element. Hence it does not have red eyes. Such flies were not used for further tests. This is the explanation for the phrase, "Non-Third,
Table 1. Scoring the GO cross for potential mutants. The data from bottles A, B, and C have been combined together.

<table>
<thead>
<tr>
<th>Bottle #</th>
<th>*w+/+1</th>
<th>*w+/Tm3, Sb</th>
<th>Mutant names</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>89</td>
<td>183</td>
<td>R7, 9, 10, 25x to 33, 46 to 48, 98 to 101, 115, 116</td>
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<tr>
<td>2</td>
<td>106</td>
<td>268</td>
<td>R18, 19, 34 to 36, 49, 50 102z to 107z</td>
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<td>3</td>
<td>49</td>
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<td>R13,</td>
</tr>
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<td>4</td>
<td>55</td>
<td>181</td>
<td>R1, 34 to 38, 51, 52</td>
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<tr>
<td>5</td>
<td>82</td>
<td>261</td>
<td>R108a to 112a, 117, 118</td>
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<tr>
<td>6</td>
<td>36</td>
<td>267</td>
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<td>30</td>
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</table>
Table 2. The R mutants were crossed with +1/Tm3. The genotype of concern is *w+/+1. The table shows the data for 30 mutants.

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<th>*w+/+1</th>
<th>*w+/Tm3</th>
<th>+1/Tm3</th>
<th>wa; Sb+/Total</th>
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<td></td>
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<td>No flies</td>
</tr>
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<td>R2</td>
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<td>3</td>
<td></td>
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<td>12</td>
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<td>Keep</td>
</tr>
<tr>
<td>R5</td>
<td>25/26 w+ dysgenic</td>
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<td></td>
<td></td>
<td>Discard</td>
</tr>
<tr>
<td>R6</td>
<td>14/15 w+ dysgenic</td>
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<td>Non III, Discard</td>
</tr>
<tr>
<td>R10</td>
<td>11</td>
<td>10</td>
<td>40/134</td>
<td></td>
<td>Discard</td>
</tr>
<tr>
<td>R11</td>
<td>10/11 w+ dysgenic</td>
<td></td>
<td></td>
<td>9/40</td>
<td>Non III, Discard</td>
</tr>
<tr>
<td>R12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Keep</td>
</tr>
<tr>
<td>R13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>This mutant was lost</td>
</tr>
<tr>
<td>R14</td>
<td></td>
<td></td>
<td>4/28</td>
<td></td>
<td>Non III, Discard</td>
</tr>
<tr>
<td>R15</td>
<td>38/45 w+ dysgenic</td>
<td></td>
<td></td>
<td></td>
<td>Discard</td>
</tr>
<tr>
<td>R16</td>
<td>3/4 w+ dysgenic</td>
<td>33</td>
<td></td>
<td>22/61</td>
<td>Non III, Discard</td>
</tr>
<tr>
<td>R17</td>
<td>44</td>
<td>39</td>
<td>4/113</td>
<td></td>
<td>Keep</td>
</tr>
<tr>
<td>R18</td>
<td></td>
<td></td>
<td>22/91</td>
<td></td>
<td>Discard</td>
</tr>
<tr>
<td>R19</td>
<td></td>
<td></td>
<td>42/137</td>
<td></td>
<td>Discard</td>
</tr>
<tr>
<td>R20</td>
<td>41</td>
<td>42</td>
<td>11/130</td>
<td></td>
<td>Keep</td>
</tr>
<tr>
<td>R21</td>
<td>40</td>
<td>38</td>
<td>0</td>
<td></td>
<td>Keep</td>
</tr>
<tr>
<td>R22</td>
<td>43</td>
<td>33</td>
<td>0</td>
<td></td>
<td>Keep</td>
</tr>
<tr>
<td>R23</td>
<td>48</td>
<td>31</td>
<td>0</td>
<td></td>
<td>Keep</td>
</tr>
<tr>
<td>R24</td>
<td></td>
<td></td>
<td>28/149</td>
<td></td>
<td>Discard</td>
</tr>
<tr>
<td>R25x</td>
<td></td>
<td>7</td>
<td>2</td>
<td>17/61</td>
<td>Discard</td>
</tr>
<tr>
<td>R26x</td>
<td></td>
<td>33</td>
<td>31</td>
<td>5</td>
<td>Keep</td>
</tr>
<tr>
<td>R27x</td>
<td></td>
<td>25</td>
<td>30</td>
<td>7</td>
<td>Keep</td>
</tr>
<tr>
<td>R28x</td>
<td>4/5 w+ dysgenic</td>
<td></td>
<td></td>
<td>23/73</td>
<td>Discard</td>
</tr>
<tr>
<td>R29x</td>
<td></td>
<td></td>
<td>28/98</td>
<td></td>
<td>Discard</td>
</tr>
<tr>
<td>R30x</td>
<td>63</td>
<td>45</td>
<td></td>
<td></td>
<td>Keep</td>
</tr>
</tbody>
</table>

Discard” in the last column in Table 2. Each mutant male was crossed with 3 virgin females and their progeny was scored. Only 3 types of progeny were recovered since the individual homozygous for the deficiency, +1/ +1 are not viable. The 3 types of recovered progeny were *w'/ +1 (red eyes, long bristles), *w'/ TM3, Sb (red eyes, stubble bristles),
and +1/TM3, Sb (orange or white apricot eyes, stubble bristles). 260 mutants from the G0 cross in the R batch were scored similarly. For the R batch, 5,401 chromosomes were screened per bottle. In Batch S, 32 bottles were set up and sub cultured 3 times to give 96 bottles. 105 mutants were collected from these and crossed to +1/TM3.

6,461 chromosomes were screened per bottle in the S batch. An average of 63.77 chromosomes were screened per bottle. The results are shown in Table 3. From these 606

Table 3. The total number of batches and the number of bottles scored in each batch.

<table>
<thead>
<tr>
<th>Batch Name</th>
<th>No. of bottles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch A</td>
<td>12</td>
</tr>
<tr>
<td>Batch B</td>
<td>12</td>
</tr>
<tr>
<td>Batch C</td>
<td>12</td>
</tr>
<tr>
<td>Batch D</td>
<td>12</td>
</tr>
<tr>
<td>Batch E</td>
<td>12</td>
</tr>
<tr>
<td>Batch F</td>
<td>42</td>
</tr>
<tr>
<td>Batch J</td>
<td>24</td>
</tr>
<tr>
<td>Batch H</td>
<td>27</td>
</tr>
<tr>
<td>Batch JG</td>
<td>30</td>
</tr>
<tr>
<td>Batch L</td>
<td>102</td>
</tr>
<tr>
<td>Batch M</td>
<td>135</td>
</tr>
<tr>
<td>Batch R</td>
<td>90</td>
</tr>
<tr>
<td>Batch S</td>
<td>96</td>
</tr>
<tr>
<td>Total = 13 batches</td>
<td>Total = 606 bottles</td>
</tr>
</tbody>
</table>
bottles, a total of 38,645 chromosomes were screened. The potential mutants were collected and numbered individually. Single pair matings were established and in this manner the progeny of approximately one thousand flies were screened individually.

*The L and M batches*

For L and M batches, female dysgenic flies were used in the G0 cross. The female flies were PGR848w'/ryPΔ2-3wc.ry' and had transposase activity. These females were crossed to males that were +1/ TM2, Ubx. The progeny of these flies were of four types (described previously). Among them, the *w'/ +1 flies (males) were collected and crossed to virgin females carrying the deficiency chromosome, OF4. Since the TM2/ TM2 combination is lethal, only the genotypes of the 3 viable progeny are shown in Table 4. Among flies with the genotype, *w'/ OF4 some are inviable. This indicates an insertion of the P element in the region covered by the deficiency. Since the OF4 deficiency spans the Bpt region, such flies are of interest to us. Hence the comment “Keep” is found in the last column indicating that such mutants were retained for further screening. The L batch consisted of 102 bottles, each having 50 females and 50 males. A total of 143 mutants were collected in this batch for the next cross. The sibling flies, *w'/ TM2, Ubx were identified by the Ubx marker on the Tm2 balancer. This mutation makes the halteres slightly bigger than the wild type. This phenotype is very difficult and tedious to score. Hence, for the next batches, the mutagenized chromosomes collected over +1 chromosome from the G0 cross was mated again with +1/TM3, Sb flies. From this cross, we could stabilize the mutagenized chromosome over the TM3, Sb balancer and also have more flies available to do the next set of crosses. In the L and M batches, there were many flies with mosaic eyes indicating that they have continuing transposon activity. These are called dysgenic and Table 5 gives data regarding the number of mutants versus number of dysgenic siblings. Dysgenic siblings from all the 102 bottles of L batch were noted in this manner. For the next batch, M, female dysgenics were used again. The number of
Table 4. The mutants *w+/*1 scored from 102 bottles of L batch were crossed to the deficiency OF4/ Tm2, Ubx. Data from 30 mutants is shown.

<table>
<thead>
<tr>
<th>Mutant name</th>
<th>*w+/ OF4</th>
<th>*w+/ Tm2, Ubx</th>
<th>.+1/ Tm2, Ubx</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>17</td>
<td>26</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>20</td>
<td>26</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>L3</td>
<td>41</td>
<td>24</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>L4</td>
<td>13</td>
<td>21</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>L5</td>
<td>9</td>
<td>7</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>L6</td>
<td>15</td>
<td>21</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>L7</td>
<td>34</td>
<td>47</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>L8</td>
<td>2</td>
<td>10</td>
<td>8</td>
<td>Keep</td>
</tr>
<tr>
<td>L9</td>
<td>40</td>
<td>39</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>L10</td>
<td>No flies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L11</td>
<td>12</td>
<td>17</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>L12</td>
<td>15</td>
<td>15</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>L13</td>
<td>0</td>
<td>4</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>L14</td>
<td>22</td>
<td>34</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>L15</td>
<td>17</td>
<td>23</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>L16</td>
<td>9</td>
<td>21</td>
<td>12</td>
<td>Keep</td>
</tr>
<tr>
<td>L17</td>
<td>29</td>
<td>4</td>
<td>111</td>
<td>Keep, 4 *w+/OF4 and 3 +1/Tm2 had patch</td>
</tr>
<tr>
<td>L18</td>
<td>11</td>
<td>7</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>L19</td>
<td>No flies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L20</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>L21</td>
<td>17</td>
<td>44</td>
<td>31</td>
<td>Keep</td>
</tr>
<tr>
<td>L22</td>
<td>12</td>
<td>57</td>
<td>46</td>
<td>Keep</td>
</tr>
<tr>
<td>L23</td>
<td>5</td>
<td>7</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>L24</td>
<td>21</td>
<td>49</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>L25</td>
<td>8</td>
<td>30</td>
<td>42</td>
<td>Keep</td>
</tr>
<tr>
<td>L26</td>
<td>12</td>
<td>17</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>L27</td>
<td>27</td>
<td>55</td>
<td>37</td>
<td>Keep</td>
</tr>
<tr>
<td>L28</td>
<td>12</td>
<td>11</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>L29</td>
<td>7</td>
<td>13</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>L30</td>
<td>6</td>
<td>2</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>
Table 5. The number of dysgenic siblings per bottle in the L batch is shown. Data from 30 bottles are shown.

<table>
<thead>
<tr>
<th>Bottle name</th>
<th>No. of mutants (*(w^+/+1))</th>
<th>No. of dysgenic siblings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 A, B, C</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>2 A, B, C</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>3 A, B, C</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>4 A, B, C</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>5 A, B, C</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>6 A, B, C</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>7 A, B, C</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>8 A, B, C</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>9 A, B, C</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>10 A, B, C</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

dysgenic progeny from all the 135 bottles were counted. Forty dysgenics from the G0 cross of the M batch were analysed in detail. The continued dysgenesis may be because a) the P element jumped to the P\(\Delta2\)-3 chromosome or b) the egg carries the transposase in the cytoplasm causing somatic jumps. If the first hypothesis is true, the progeny of these dysgenic flies will be dysgenic also. But if it is the cytoplasm carrying the transposase, then in the next generation the effect of the transposase is diluted and we should see dysgenic and non-dysgenic flies or reduced dysgenesis. Forty dysgenic males carrying the mutagenized chromosome, \(*\!w^+/\!TM3, Sb\) was crossed with \(+1/\!TM3, Sb\) virgin females. A sample of the data obtained is shown in Table 6.
Table 6. Analysis of dysgenic siblings collected from the G0 cross of the M batch. Forty dysgenic males, *w+/Tm3 were crossed to +1/Tm3 virgin females. Data from 10 crosses is shown. #D refers to the number of dysgenics scored in this cross.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>*w+/+1</th>
<th>*w+/Tm3</th>
<th>+1/Tm3</th>
<th>wa, Sb+</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>#D</td>
<td>#D</td>
<td>#D</td>
<td>#D</td>
<td>#D</td>
</tr>
<tr>
<td>Dsm1</td>
<td>1</td>
<td>22</td>
<td>1</td>
<td>50</td>
<td>34</td>
</tr>
<tr>
<td>Dsm7x</td>
<td>7</td>
<td>7</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Dsm8x</td>
<td>2</td>
<td>2</td>
<td>11</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Dsm9</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Dsm10</td>
<td>17</td>
<td>14</td>
<td>10</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>Dsm13</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Dsm14</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Dsm19</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Dsm20</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Dsm34</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

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Table 7. Preliminary characterization of the 19 P insert alleles in the *Bpt* region.

<table>
<thead>
<tr>
<th>Name of mutants</th>
<th>B9, B18x, M51, M56, M75, M135, S7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recessive lethal inserts</td>
<td>M65, M107, M182, S10, S12, S22, S28, S36, S45</td>
</tr>
<tr>
<td>Inserts that are not lethal</td>
<td></td>
</tr>
<tr>
<td>Others (not tested)</td>
<td>H47, M105A, M130</td>
</tr>
</tbody>
</table>

**Discussion**

Positional cloning and transposon tagging were two approaches to isolating the *Blackpatch* region. The YAC, NO7.75 spans the 92A6 to 92B1 region containing *Blackpatch*. The approach was to use PFGE to resolve the YAC, isolate YAC DNA from the gel, make a sub-genomic library of the DNA and isolate *Bpt* within the library. Variations of this approach have been used to clone mammalian genes. The bottleneck in this approach is to be able to isolate a large quantity of DNA to make a library. The problems in the approach are many. It takes a large quantity of very dense yeast culture to make the PFGE runs feasible. A stationary yeast culture of 50 to 100 mls, under the best culture conditions takes more than a day. Since the yeast is lysed within the agarose plugs, it is not possible to check for lysis prior to running the PFGE. So this step of the procedure cannot be tested. Errors at this step will not be detected until after the PFGE. The gels are large and difficult to handle. The type of agarose that is used, SeaKem GTG which is useful for isolating the DNA later also makes the gel fragile and a frequent problem is breakage and fragmentation of the gel. Every PFGE gel must be run very slowly (fourteen to twenty-four hours) and consequently takes a long time. The whole
procedure from growing the yeast cells to resolving the YAC on the gel takes more than a week and the frequency of a successful PFGE run was only one in four. Eventually, the amount of YAC DNA isolated from two gels (four lanes each) was 0.55 ug of DNA. At this rate, if the procedure worked perfectly every time, it would take twenty weeks (upto 5 months) just to obtain 10 ug of DNA. The amount of time and effort to obtain enough YAC DNA to proceed with the library construction and subsequent cloning was too great to make this approach practical. The literature supports the fact that YACs are impractical vectors for cloning *Drosophila* genes. The PI clones are easier to handle and seem to be a more feasible approach to cloning *Bpt*.

The transposon tagging yielded 19 inserts in the *Bpt* region (92A6 to 92B1). 38,645 chromosomes were screened to obtain these inserts. The average number of chromosomes that are screened for such a mutagenesis is 10,000. This is three times the recommended number for a mutagenesis screen. P elements have variable frequency of insertion for different genes. The *singed* locus is very amenable to P inserts and the frequency of inserts is as high as 1x10^6 (Hawley, et al., 1988). This is unusual and the expectation of P insertion frequency is between 10 to 50% of the time. Certain loci may be truly refractory to P insertions. But the true molecular reasons for these variations is not clear. Heterochromatic regions are considered more refractory than other regions but P. Zhang from A. Spradling's laboratory has successfully isolated P element insertions in the heterochromatic region (Zhang and Spradling, 1994). The problem may also be in having an effective screen to isolate the insertion event. For practical purposes, P element insertion events are considered "random" since all the molecular factors affecting the event are uncertain. A common method of increasing the frequency of obtaining an insertion in the gene of choice is the use of multiple P elements, a "machine gun" approach. Jack Girton screened for revertants of the dominant black patch phenotype of *Bpt* using the
Birmingham chromosome containing 13 P elements as the "jump-starter". This screen also did not result in any insertions within the \textit{Bpt} gene.

It was suggested by B. Engels and W. Bender that female dysgenics should be used in the GO cross since they had some preliminary evidence to show that the frequency of P element transposition was higher in the germline of female dysgenics. Consequently, the possibility of obtaining an insertion in the gene of interest must be higher. Fifteen thousand chromosomes were screened using female dysgenics (flies in which the P element has been mobilised). A P element insertion in \textit{Bpt} was not found. A problem with using female dysgenics is that the P element continues to be active in the progeny. An essential requirement of the P element mutagenesis scheme is the control on the activity of the P element. It is very important that the P element transposition event in the progeny of the dysgenic remain stable. To ensure this, the PA2-3 is crossed out so that no more transposase activity is available and the insertion becomes a stable event. Only then, the event can be screened in the subsequent crosses. In the progeny of the female dysgenic, it is possible that the transposase enzyme is packed into the cytoplasm of the egg. Thus it becomes available once again for P activity. The higher frequency of transposition of the element is offset by "continued" activity of the P.

There is preferential transposition of P elements to nearby chromosomal sites (Tower, Karpen, Craig, and Spradling, 1993). They report that there is a 43 to 67 fold increase in transposition events within 0 to 128 Kb from the starting point. The \textit{Bpt} region is between 92A6 to 92B1 and occupies about 120Kb. The screen was set up to recover insertions within this 120Kb region. The frequency of transposition in our experiments, for this region was 0.049\% (19/38,645 x100). This does not prove or disprove the idea put forward by Tower et.al. But the lack of inserts in \textit{Bpt} suggests that the idea of "hot sports" for insertion events may be true and \textit{Bpt} may not be a favourable site of insertion. Also the idea of "local" transposition varies. Three insertions into the white locus occurred...
at the same nucleotide (O'Hare and Rubin, 1983). Local may mean within a few base pairs away as opposed to the idea of Tower et. al.

What are some of frequencies established for P element insertions into Drosophila genes? A review by Engels provides the following info. They suggest 0.25 insertions per element-generation. With defective P elements, Robertson obtained a transposition rate of 1.82 (Robertson, et al., 1988). With marked P elements the rates are highly variable between 0.01 and 0.2 with an average of 0.03.

We conclude that a thorough investigation of the possibility of tagging the Bpt region using P elements has been conducted. Bpt may be refractory to P elements due to special characteristics in its structure. Cloning the Bpt gene using P1 clones that span the region seems to be a more direct and feasible approach. The P element insertions in the Bpt region will be useful as reference points in the chromosomal walk that will be conducted using the P1 clones.

References


In this study, we examined the effects of prolonged exposure to carbon dioxide on the electrical activity in the visual system of *Drosophila*. Electroretinograms (ERG) were measured in *Drosophila* after exposure to carbon dioxide followed by a lengthy time period for recovering from anesthesia. Previous studies on the effect of anaesthetics on electroretinograms have focused primarily on ether. The studies noted that ether and carbon dioxide cause temporary alterations in ERGs during exposure (Stark, 1972). We show statistical evidence that the amplitude and the shape of the electroretinogram is not altered permanently by prolonged exposure to carbon dioxide.

The electroretinograms in this study have been characterized in terms of amplitude and shape. The normal ERG has quick responses to the beginning and ending of the light stimulus. These are the “on” and “off” transients due to electrical activity in the synaptic junctions with the L1 and L2 cells of the lamina. The sustained corneal negative is due to the electrical activity in the retina (Hotta and Benzer, 1969). The amplitude or height of the ERG is the distance between the tips of the “on” and “off” transients. The amplitude of the ERG is measured as mV/Division. In the figure, one division is seen as one large square with twenty five small squares.
The ERGs were measured in y w sn flies. Flies with the white mutation are used instead of wild type flies to measure ERGs because the white eyes do not 'light adapt' as quickly as the red eyes. ERGs were also measured from w "fa" and w "fa"; Bpt mutants. w "fa" mutants have apricot coloured eyes and rough facets. In w "fa"; Bpt mutants, the rough eyes have a black patch on the retina. This is due to death and degeneration in the retina and the underlying optic lobes (Duus et. al., 1992). This study reports the results from measuring ERGs in y w sn flies when exposed to varying levels of carbon dioxide.

Flies were prepared for measuring ERG in batches. Flies in each batch were anesthetized with CO2 and glued to a coverslip with nail polish. Each batch contained flies exposed to 3 min, 5 min, and 8 min of carbon dioxide. The coverslip was laid on a block of agar, prepared with 0.9% NaCl, and the flies were connected to the block with small strips of agar. A silver reference electrode was inserted into the block of agar, and the signals from the bodies of the flies (background noise) was grounded. A fiber optic illuminator provided a white light stimulus that was focused onto the eyes of the fly by using a mirror and a convex lens. Wavelength filters of 470 nm and 568 nm were used to change the wavelength of the stimulus. The light stimulus always lasted for one second and is shown as a black bar in the figure. The average amplitude of the ERG was measured at two wavelengths, 470 nm and at 568 nm. Electroretinograms were measured from each fly by inserting a recording electrode filled with 0.9% NaCl into the eye. Signals from the recording electrode were amplified 10x by a preamplifier, Dagan corporation (Minneapolis, MN) and then displayed on the screen of an oscilloscope. The recording electrodes were made by using glass capillaries with filament, No. TW100F-3, World Precision Instruments, (Sarasota, FL).

The electroretinograms always showed the on and off transients as seen in Figure 1. Statistical analyses were done on the amplitudes of the ERGs recorded from y w sn flies. Seven flies, exposed to 3 min of carbon dioxide show an average
Figure 1. Electoretinogram of a y w sn fly after anaesthetizing with carbon dioxide. The ERG shows the presence of laminar transients and retinal negative. It was measured at 0.1 V/D and a speed of 1 sec/D. The stimulus lasted for 1 sec and is shown as a black bar.
amplitude of 8.21 mV (470nm) and 8.14 mV (568nm). Five flies, when exposed for 5 min have average amplitudes of 6.1 mV (470nm) and 5.9 mV (568nm). Even after exposure to carbon dioxide for 8 min, ten flies show amplitudes of 5.8 mV (470nm) and 5.6 mV (568nm). The differences between the means is not significant (P > 0.5).

In our study, we have conclusively shown that prolonged exposure to carbon dioxide does not alter the amplitude and shape of the ERGs permanently. Previous studies have measured the effects of anaesthetics on electroretinograms. Prolonged exposure to ether for more than 3 minutes permanently blocked the transients and sometimes, even lowered the corneal negative. Measurement of ERGs during short exposure to carbon dioxide showed reversible changes. The off-transient disappeared in less than 5 seconds and the on-transient disappeared in less than 10 seconds (Stark, 1972). These studies, taken together, indicate that the metabolic and synaptic effects of these two anaesthetics on electroretinograms are very different. We suggest that carbon dioxide should be the anaesthetic of choice when measuring electroretinograms.

References

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CHAPTER 6. GENERAL CONCLUSIONS

Development of a multicellular tissue is a complex process involving the action of several interacting genes in pathways. Genetic analyses of the development of tissues and organs addresses the questions of cell determination, differentiation and pattern formation and in particular, the identity and function of the genes that control these processes. The adult visual system of *Drosophila* is a good model system to study the genetic control of the developmental processes. The retina and the optic lobes develop from different tissues and development is completed only after complex interactions between the tissues are established. *Blackpatch* is a unique gene that is functioning at this time of tissue interactions. Analyses of the *Blackpatch* mutation will not only answer questions in the events of pattern formation of the optic lobe but also seminal questions regarding the role of genetic pathways in these interactions and finally how these dialogues function in the development and function of an organism.

The characteristics of the *Blackpatch* mutation provide a basis for its role in visual system development. The mutation causes cell death only in the lamina and the retina. Several lines of evidence provided by the ERG analysis, cell death analysis, somatic mosaics and disc transplant analysis supports this conclusion. The cell death in the lamina takes place in a specific time period in development between 42 to 50hrs pp. This spatial and temporal specificity indicates that *Bpt* function in visual system development is required only during a certain time in specific tissues. The cell death is apoptotic in nature indicating that normal *Bpt* function is required for the correct functioning of the programmed cell death pathway. *Blackpatch* shows an interaction with *Notch*, a transmembrane protein that is required in various developmentally important signal transduction pathways. These characteristics of spatial and temporal specific expression, apoptotic cell death in mutants and interaction with signal transduction pathways are reminiscent of
neurotrophic genes. Based on the experimental evidence for the characterization of \( Bpt \) and its similarities to neurotrophic genes in other systems, we propose that \( Blackpatch \) is a gene in a neurotrophic pathway. Knowing the tendency to parsimony in nature, it is not too much speculation to expect the existence of neurotrophin or neurotrophin-like genes in invertebrate systems.

The events that have been described so far: the cell death evidence by trypan blue, the visible degeneration, the temperature sensitive period of requirement of \( Notch \) (\( facet-glossy \)) and the sequence of normal developmental events can be comparatively viewed on a time scale shown in Figure 1. During its development, the retina and optic lobes commence an interaction when the retinal axons reach their targets within the lamina. This takes place between 30 to 46 hours post pupariation (pp). A period of normal programmed cell death overlaps these events as it is seen between the beginning of pupariation and 42 hours pp. In the \( Bpt \) mutants, the lamina shows greater amounts of death compared to the wild type. This is seen starting at 36 hours pp with a mid point at 44 hours post pupariation. The mutant retina shows large amounts of death earliest at 58 hours with a sharp peak at 60 hours post pupariation. The degeneration and blackening that gives the visible phenotype takes place between 68 to 72 hours pp.

There is overwhelming evidence that the focus of the \( Bpt \) mutation and the most probable location of gene action is the lamina. This spatial specificity taken together with the sequence of events at that time indicate that \( Bpt \) is needed in the lamina at a time when they are receiving innervation from the retinal axons. \( Bpt \) is not needed later for the function of the visual system. It is necessary for the structural development and maintenance. The further analysis of the \( Bpt \) mutation will provide important information not only about the development of a complex structure like the lamina but also about the tissue interactions between the retina and lamina. It is an established fact that signals from the retina are necessary for the complete development of the lamina. There is also some
Figure 1. A diagram showing the time of $Bpt$ -induced trypan blue staining with respect to developmental events in the retina and lamina.
Axons reach laminar targets

Normal retina-lamina cell death

Temperature sensitive period

facet

Bpt

Periods of trypan blue stain

Bpt retina

Bpt lamina

Normal lamina

End of pupal period
evidence for retrograde signals from the lamina for long term maintenance of the photoreceptor cells of the retina. With Blackpatch we see evidence for a more dynamic and continuous dialog between the two tissues. The death signals are communicated to the retina shortly after the laminar death. Does this imply maintenance signals from the lamina to the retina even as development is proceeding in the wild type? The interaction with Notch further strengthens the idea that Bpt is involved in some kind of signaling pathway between the laminar cells, and between the lamina and the retina.

The hypothesis explained so far is only one of several possibilities. Blackpatch and Notch could be functioning in two different pathways and the simultaneous failure of both causes the large scale death of cells. It is possible that Blackpatch function in early development is somewhat similar to its function in the visual system. Neurotrophic pathways in mammals are employed as mitogenic agents of proliferation. The lethal phase occurs at a time when the central brain and optic lobe neuroblasts are undergoing proliferation. Further analyses of the gene, especially its molecular structure and function will help to elucidate the role of Bpt further.