Development in vitro of the visual system in Drosophila melanogaster

by

Chinglu Li

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
at
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To dew lovers
Frontispiece

Structural organisation of the adult visual system of the compound eye and optic lobe of Drosophila melanogaster, seen in horizontal section stained by the Bodian method. The ommatidial array in the retina (R) innervates the lamina (L) and, via the external chiasma (X), the distal part of the medulla (M). This in turn connects with the inner neuropils, the lobula (Lo) and, posteriorly, the lobula plate (Lp). The medulla comprises two parts, distal (d) and proximal (p). The retina is detached from the cornea (C) through shrinkage during fixation. AN: antennal nerve; a: anterior; m: medial. Scale bar 50µm. (From Meinertzhagen and Hanson, 1993.)
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Abstract

A primary culture system for the visual system in *Drosophila* has been established. With this system, neurite outgrowth from intact eye imaginal discs, eye-disc fragments and dissociated eye imaginal-disc cells, as well as from dissociated optic lobe cells has been obtained. Neurites express antigens to the insect neuronal marker, anti-HRP. Furthermore, immunoreactivity to antibody 24B10 indicates that neurites extending from eye-imaginal discs are axons of photoreceptors. Three culture media were tested for their ability to support the survival of, and neurite extension from, eye-discs in vitro at 23°C. These, with supplements, were: five parts of Schneider's *Drosophila* medium with four parts of basal Eagle's medium; Leibovitz's L-15 medium; and Shields and Sang's M3 modified medium (MM3). The best results was obtained with 2%FBS-MM3. Differentiation of screening pigment cells required the presence of 1µg/ml 20-hydroxyecdysone (20-HE) in the medium. Only donor animals of a particular age range gave appropriate eye-disc differentiation. Eye-discs from pupae 5h old (P+5) or older commenced ommachrome synthesis *in vitro* in a temporal sequence close to that found *in vivo*. Pigment synthesis was delayed in eye discs from younger flies. Drosopterin was not synthesized *in vitro* under these conditions. Eye-discs from third-instar larvae or white prepupae continued their mitotic activity, which, together with the advance of the morphogenetic furrow, is consistent with continued pattern formation *in vitro*.

Metamorphosis of flies is initiated by a surge in the haemolymph titre of ecdysteroids. Even though all cells in the eye discs are exposed to this hormone surge, cellular differentiation is variously susceptible to it. Immunoreactivities to anti-HRP, 22C10 and 24B10 have all been detected in eye-discs cultured with (1µg/ml) or without 20-HE, suggesting that the differentiation of neuronal and photoreceptor antigens does not require 20-HE. Formation of ommochromes, corneal lenses and bristles were detected in P+5 eye-disc cultures with 1µg/ml 20-HE, but not those lacking the hormone. The differentiation of synaptotagmin, and the elongation of extending photoreceptor neurites both occurred without 20-HE. Adding hormone to the culture medium increased both the number of synaptotagmin immunoreactive spots along the length of the neurites and average neurite length, however. Synthesis of histamine, the photoreceptor transmitter, occurred in eye-disc cultures. Combined with the expression of synaptotagmin, this suggests that photoreceptor axons may release transmitter. Most (>72%) neurite outgrowth seen in cultures was from photoreceptor axons that regenerated but new ommatidial differentiation of neurites also occurred in culture. These neurites were able to form synapse-like contacts with neighbouring cells, as seen in EM. This recommends the potential for tissue culture as a system to study synaptogenesis.
List of Abbreviations and Symbols

° degree
°C degree Celsius
% percent
/ per
> more than
< less than
μg microgram
μl microlitre
μm micrometer
20-HE 20-hydroxyecdysone
BrdU 5-bromo-2′-deoxyuridine
CNS central nervous system
d day
DAB 3,3′-Diaminobenzidine
DIC differential interference contrast
disco disconnected
DIV day in vitro
dH2O distilled water
DNA deoxyribonucleic acid
EDCDI l-emyl-3-(3-dimethylaminopropyl) carbodiimede
EDTA ethylenediamine-tetraacetic acid
EH eclosion hormone
FBS fetal bovine serum
FITC fluorescein isothiocyanate
g  gram
H₂O₂  hydrogen peroxide
HNMT  histamine-N-methyltransferase
h/hr.  hour
HRP  horse-reddish peroxidase
JH  juvenile hormone
kd  kilo dalton
m  meter
M  mole
mM  millimole
min.  minute
mg  milligram
ml  millilitre
mm  millimetre
MM₃  Shields and Sang’s M3 modified medium
NGF  nerve growth factor
NGS  normal goat serum
nm  nanometre
PBS  phosphate-buffered saline
PTTH  prothoracicotropic hormone
SEM  scanning electron microscopy
TBS  tris-buffered saline
TEM  transmission electron microscopy
T₁/₂  half time
TRITC  tetramethylrhodamine-isothiocyanate
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Chapter 1
INTRODUCTION

The structural and functional complexity of the nervous system is as much a triumph of morphogenesis as it is a genetic extravagance. To understand how any nervous system functions and develops poses a major challenge to neurobiologists. This is due in part to the presence of the large number of cells in the nervous system (about an estimated $10^{12}$ neurons and at least $10^{13}$ glial cells in the human brain, Williams and Herrup, 1988) and in part to the great phenotypical diversity of the cell types. The astronomic number and diverse types of neurons are moreover not mixed randomly, but are structurally highly organized. The proper function of the nervous system depends on the organized and highly specific wiring among the neurons, and this in turn depends on the orderly array of neurons into strata and assemblies. For a given species, these specific wiring patterns are reproducible, or in other words, are developmentally programmed — the neurites of each of the billions of neurons unerringly find their targets among the seemingly confusion of the "jungle" of other neurites and cells, during the development of the nervous system.

The chief questions in nervous system development can be asked at several levels, each of which assumes prominence of a particular step in the sequences of development. First, how do cells become committed to a neuronal fate? In both vertebrates and invertebrates, the nervous system is derived from non-neuronal primordia. Is the neuronal fate determined by an intrinsic developmental program or by extrinsic developmental signals such as cell-cell interactions? At which developmental step does the commitment to a particular fate take place? Second, how does a cell then manifest its commitment to a neuronal fate by differentiating into one of the many different neuronal phenotypes? What range of possible cell types can it adopt? Third, how does a neuron send out its processes to far distant sites to find its proper target and with that target form functional synapses?
Invertebrate animals have contributed greatly to our present knowledge of developmental neurobiology. Even though they are of early ancestry in the evolution of nervous system, and are often morphologically less complex, the nervous systems of invertebrate have many similarities to those of vertebrates. This is especially true at the cellular and molecular levels, rather than at a morphogenetic level. For example, the mechanisms underlying long-term sensitization in *Aplysia* are very similar to those involved in learning and memory in vertebrates (Bailey and Chen, 1989) and some of the biochemical and molecular events involved in both invertebrate and vertebrate employ similar serotonin receptor mechanisms (Fagnou and Tuchek, 1995). Parallel structures can be identified in a comparison between the synaptic circuits of the vertebrate retina and the fly's optic neuropils, however (Meinertzhagen, 1993), which exhibit similarities in the two evolutionarily distant animal groups, despite obvious differences in the gross structures of their visual systems. These similarities are also functional, despite the fact that the vertebrate retina and the compound eye do not share a common developmental origin (Meinertzhagen, 1996). In an alternative view, however, the Pax-6 gene is thought to regulate the fate of photoreceptors in all animal groups (Halder et al., 1995). Genetic studies of neuronal development in the nematode *C. elegans* (Sulston and Horvitz, 1977; Sulston and White, 1980) and in the fruit fly *Drosophila* (Campos-Ortega, 1988) have revealed specific ways in which the mechanisms governing the determination of cell fate are both intrinsic and extrinsic, and these have provided insights into the study of more complex vertebrate systems. More and more recent evidence at the molecular level has indicated the ways in which similar molecules are used by vertebrate and invertebrate animal to construct their nervous systems (Harrelson and Goodman, 1988; VanBerkum and Goodman, 1995), presumably, because these have been inherited from a common ancestor.

The nervous systems of invertebrates not only provide meaningful models for counterpart systems in vertebrates, they also embody several advantages over the vertebrate
nervous system for studies devoted to neuronal development. The small number of cells in invertebrates (compare the estimated $10^{12}$ neurons in the human nervous system (Williams and Herrup, 1988) with the estimated $3.4 \times 10^5$ neurons in Musca (Strausfeld, 1976) makes the nervous systems of most invertebrates a numerically less complex challenge in both architecture and function. This advantage facilitates the isolation of a phenomenon of particular interest. The cells of the invertebrates are also more accessible for observation and manipulation than those of vertebrates. In many vertebrate animals, neurogenesis and the structural determination of the nervous system occur early in animal development, mainly during embryonic development. This makes the developmental study of nervous systems difficult, especially in mammals (in which development occurs in utero). By contrast ancestral vertebrates, such as fishes and amphibians, as well as many invertebrates, such as insects, undergo relatively little embryonic development before the egg hatches into the juvenile. Metamorphosis, which is a series of developmental steps by means of which animals such as insects pass from the larval form into the adult, occurs during postembryonic development and is responsible for the mature form of the adult. There are many resemblances between postembryonic and embryonic development, a feature which enables the study of neural development to be postponed until later during development.

The availability of powerful and sophisticated genetics methods in Drosophila and the relatively simple anatomy of the adult together have made Drosophila a very significant model for many recent studies on neural development. A number of related approaches have isolated neurological mutants, and these have been used to identify the genes for specific features of neurogenesis. For example, a group of so-called neurogenic mutants have been used to elucidate the mechanisms of cell fate commitment in early neuroembryogenesis (Campos-Ortega, 1988). In a second example, structural mutants of the brain that cause cell death amongst particular cell populations simplify the structure of the brain and allow specific behavioral deficits to be correlated with the loss of identified
neurons (Heisenberg et al., 1978). Clear examples in the optic lobe have permitted the establishment of relationships between optic lobe structures and specific optomotor behaviors (Fischbach et al., 1989). In a third approach, the instrumental use of genetic mosaics has been made to study cell lineage (Ready et al., 1976; Lawrence and Green, 1979), and to construct genetic "fate maps" that can offer information about the embryonic origins of particular adult structures (Hotta and Benzer, 1972), as well as to determine the cell autonomy of gene action (Becker, 1976).

The visual system is the best studied system in the *Drosophila* brain. Even though the compound eyes occupy a large area of the head, and the optic lobes contribute a large volume of the nervous system (Hinke, 1961) to process visual information, the visual pathway is built on a modular principle. From the retina to the underlying neuropils, the architecture reflects an iterated composition (Meinertzhagen, 1993) that originates in the ommatidia of the retina (Meyerowitz and Kankel, 1978). Each module has a small, fixed number of cells (Meinertzhagen, 1993), at least in the retina and the underlying neuropile of the lamina, so that we can analyze the visual system by analyzing just one of the modules (Ready, 1989). Structurally, the visual system is highly ordered. Mutations affecting the peripheral visual system, especially the development of the compound eye, are therefore easily to be detected from perturbations in the pattern of eye facets.

A further reason why the development of the visual system is of such wide interest lies in the developmental potential of the thin epithelial pouch that gives rise to the eye, the eye-imaginal disc. The eye imaginal discs of a third-instar larva contain a histologically homogeneous cell population. During postembryonic development, this homogeneous population differentiates into various organs comprising a diversity of cell types, such as the different cell types found in the adult compound eye (see below), or in certain other head structures (Haynie and Bryant, 1986). Some of the cell types, such as the photoreceptors of the eye, differentiate axons and synaptic organelles, thus acquiring a neuronal phenotype, so that understanding the mechanisms governing differentiation
amongst eye imaginal disc cells will shed light on our understanding of the mechanisms that control the development of the nervous system. As a particular example, the identification of the tyrosine kinase, Ras 1, cascade of intracellular signalling that results from interactions between two cells in the assembling ommatidium, R7 and R8, provides the first important example of the molecular steps involved in neuronal determination (Krämer et al., 1991; Banerjee and Zipursky, 1990; Dickson, 1995). By this pathway, R7 becomes committed to a neuronal fate.

Another alternative to simplify the study of the developmental interactions in a nervous system under investigation is to use in vitro approaches. Tissue culture facilitates observation of development events which are difficult to see in vivo. It also permits experimental control over the spatial and temporal aspects of neural development. These considerations account for the growing use of in vitro techniques, and their application to nearly all aspects of studies concerning the structural, biochemical and electrophysiological properties of neurons.

The objectives of this thesis are to establish a primary culture system for the visual system of Drosophila melanogaster and thus to bring advantages of in vitro accessibility to this system. A variety of developmental questions that have already been identified in the development of both the eye (Wolff and Ready, 1993) and the optic lobe (Meinertzhagen and Hanson, 1993) in vivo can then be studied at the cellular and molecular level in vitro. The second part of the thesis uses the culture system to study the hormonal influences on cellular differentiation of the developing compound eye. Environmental influences on photoreceptor differentiation other than those of hormones, have been studied further, and the potential use of tissue culture to study synapse formation, aspects which will be presented in the third part of the thesis.
I. The visual system in *Drosophila melanogaster*

1.1 *Drosophila* are holometabolous insects

Insects have a number of different life-cycles, variations in the pattern by which an adult insect gives rise to the next generation of adults. Three groups of insects that can be distinguished on the basis of this variation are ametabolous, hemimetabolous or holometabolous forms of the life-cycle. In ametabolous insects, the young postembryonic stage of the insect appears as a miniature of the adult body form. Insects in this group, such as silverfish, do not go through any metamorphosis in their life-cycle. In hemimetabolous insects, the body structure of the young stage is very similar to that of the adult insect, and undergoes only a relatively minor metamorphosis in passing from the final larval stage to the adult. The dragonfly is a representative hemimetabolous insect. Distinctions between these two groups are more quantitative than qualitative and differ depending on the body organ system involved. The development of wings for example is often deferred to the final adult stage.

In holometabolous insects, such as *Drosophila*, the habits and the general structure of the body during the young stages differ greatly from the adult. As a result, holometabolous insects always undergo a dramatic metamorphosis, and a new larval stage is added to the life cycle, with an intermediate stage, the pupa, being interposed between the larva and the adult. It is at the pupal stage that most of structures, such as wings, legs and the compound eyes start to appear in their adult form. Much of the brain, too, undergoes dramatic growth and restructuring during pupal metamorphosis (Truman 1990).

1.2 The visual system of *Drosophila*

The visual system of insects consists of the compound eye (comprising the retina of photoreceptors and pigment cells, and various dioptric cells) and the underlying optic lobe. The development of the adult visual system in holometabolous insects is very different.
from that in other insect groups. Unlike ametabolous and hemimetabolous insects, in which the compound eyes attain their adult form (if not their size) in the egg, the compound eyes of holometabolous insects develop postembryonically, during the final larval and pupal stages. The structure and development of the compound eyes and the optic lobes of insects in general has been summarized by Trujillo-Cenóz (1985). Thorough reviews of the development of the compound eyes (Wolff and Ready, 1993) and optic lobes (Meinertzhagen and Hanson, 1993) in *Drosophila* are also available. A brief introduction to the development of *Drosophila* visual system is given here, as background for the work to be reported in this thesis.

The visual system of *Drosophila* contains the retina and the optic lobe which, in turn, comprises three orders of neuropil, those of the lamina, medulla and lobula complex (Frontispiece). The organization of the visual system is highly ordered. From the most distal retina to the most proximal lobula complex, the cellular organization is widely presumed to follow a modular principle (Fischbach and Dittrich, 1989; Meinertzhagen, 1993), although this is known in exact detail only in the lamina. The columnar structure of the neuropils in the optic lobe is arranged in such a way as to represent the visual world in retinotopic array (Braitenberg, 1967; Strausfeld, 1971; Meinertzhagen, 1976).

During development, the retina and its underlying neuropils are derived, independently, from different origins (Meinertzhagen, 1973; Trujillo-Cenóz, 1985). The retina arises from an epidermal epithelium, which in holometabolous insects is transformed into the hypodermal eye-imaginal discs, while the optic neuropils arise from imaginal centres in the larval brain. The establishment of the retinotopic projection of the retina therefore represents a connection between two independently developing structures.

### 1.2.1. Retina

The *Drosophila* compound eye is made up of approximately 750 to 800 unit structures, called ommatidia, which are arranged in a regular hexagonal array. The number
of ommatidia in the compound eye of the female is greater than that in the male. Each ommatidium consists of a lens system, eight photoreceptors (R1-R8) and 18 accessory cells, 12 of which are shared with neighbouring ommatidia, which together form a highly determinate assembly of 26 cells (Ready et al., 1976; Wolff and Ready, 1993).

The photoreceptors are the receptor cell component of an ommatidium and have a neuronal phenotype (axon, synapses etc). There are three portions to the photoreceptor: (1) the soma portion, bearing the metabolic machinery of the cell, as well as the photosensitive rhabdomeres, the apical surface of the photoreceptors which during development becomes transformed into an axial rod-like stack of microvilli responsible for phototransduction; (2) the axonal portion, projecting into the underlying optic neuropils; and (3) the synaptic terminal of the axon, from which photoreceptors form synaptic contacts upon their target neurons in the optic lobe (Trujillo-Cenóz, 1985).

The rhabdomeres of seven of the eight photoreceptor cells are separate, and in cross section are arranged in an asymmetrical trapezoid pattern (Dietrich, 1909; Waddington and Perry, 1960). From this pattern, the photoreceptors can be further distinguished as three different morphological types: six outer (R1-R6), and two central cells (R7 and R8). The rhabdomeres of R7 and R8 are located in tandem, R7 above R8, at the centre of the trapezoid, for which reason, R7 and R8 are referred to as the central photoreceptors (Melamed and Trujillo-Cenóz, 1968). Surrounding R7 and R8 are the larger rhabdomeres of R1-R6, the outer photoreceptors. The photosensitivity of these photoreceptors results from the photopigments (rhodopsins and xanthopsin) located in the microvilli of these rhabdomeres, each of the morphological classes of photoreceptor (R1-R6, R7, R8) with a different opsin (Pollock and Benzer, 1988). In confirmation of the three morphological photoreceptor types, eye mutants have been isolated that selectively affect the photoreceptor subtypes, for example the ora mutant, which causes the selective degeneration of R1-R6 (Harris et al., 1976). Probes to the three opsins of the Drosophila eye have also been selectively localized to particular photoreceptor classes (O'Tousa et al., 1985; Zuker et al.,
The axons of R1-R6 travel a relatively short distance to innervate the lamina, where they contact lamina monopolar and other cells, while R7 and R8 extend axons to innervate the second neuropil, the medulla, bypassing the lamina without synaptic contact (Meinertzhagen & O'Neil, 1991).

In addition to the photoreceptors of the ommatidium, there are 12 accessory cells. These include: four cone cells, two primary pigment cells, three secondary pigment cells and, shared among three ommatidia at the vertex, are three tertiary pigment cells and a mechanosensory bristle.

There are three types of pigment cells capping and surrounding the photoreceptors of each ommatidium (Waddington and Perry, 1960). The primary pigment cells sit atop the photoreceptor cells, and contain brown ommochrome screening pigment (Shoup, 1966). The secondary and tertiary pigment cells surround the photoreceptor cells, and both contain red drosopinerine screening pigment and ommochrome pigment (Wolff and Ready, 1993). The secondary and tertiary pigment cells extend to the floor of the retina, where they flatten out, together with a thin basal lamina, to form the basement membrane (Wolff and Ready, 1993). The basement membrane separates the retina from the underlying lamina cortex and neuropil.

The mechanosensory bristles develop late in pupal life, independent of the ommatidia. Their axons project to the deutocerebrum rather than to the optic lobes (Wolff and Ready, 1993).

Surmounting the entire ommatidium is the lens system, the optical component of the ommatidium. It includes the corneal lens and the pseudocone. Both are secreted by cone cells and pigment cells (Wolff and Ready, 1993). The cornea is secreted at mid-pupal development (about 70 hours after pupariation), while secretion of the pseudocone happens late in pupal life (about 110 hours after pupal development) (Cagan and Ready, 1989). The corneal lens is chitinous, while the pseudocone located beneath the corneal lens is non-chitinous.
Development of the retina

During the life-cycle of Drosophila (and other holometabolous insects), at least three sets of eyes develop: the larval eyes and the adult compound eyes and separate ocelli. The development of the photoreceptors of the larval eyes, or Bolwig's organ (Bolwig, 1946), will be mentioned later in the development of the optic lobe, because of their close relationship to the development of the adult visual pathways of the compound eye. The larval eyes degenerate in early pupal life (Ready et al., 1976). The studies reported in this thesis are concerned with events in the development of the compound eyes.

The compound eyes develop from eye imaginal discs which result from the proliferative growth of a group of embryonic cells during the larval stages. The larvae of most holometabolous insects lack typical compound eyes, and the development of compound eye structures can therefore be assigned to three developmental stages: embryonic, larval and pupal.

A. Embryonic origin of the eye-antennal discs

Imaginal discs are the primordia of most adult organs, nearly all of which develop as epidermal structures. At the cellular blastoderm stage of embryonic development, cells which will give rise to larval tissues segregate from those which will form the primordia of adult structures.

The eye-antennal discs consist of two separate portions, the eye and the antennal imaginal discs. The eye discs are the primordia of the adult compound eye and some surrounding head structures (Haynie and Bryant, 1986), while the antennal discs give rise to the adult antennae. There is no separation between the eye and antennal discs at the embryonic stage, however, and the cells in the discs still have the capability to take on either an eye-disc fate or an antennal-disc fate (Garcia-Bellido and Merriam, 1969; Wieschaus and Gehring, 1976). In higher Diptera, the eye-antennal discs originate as
lateral pouches from the deep posterior parts of the frontal sac (Poulson, 1950). By following the formation of the frontal sac Anderson (1963) traced the origin of eye-antennal discs in *Dacus tryoni* to a domain of dorsolateral ectoderm. Consistent with Anderson's results, using genetic mosaic gynandromorphs in *Drosophila*, about 23 blastoderm cells of the dorsolateral ectoderm have been estimated to be reserved for the generation of the eye-antennal imaginal discs (Garcia-Bellido and Merriam, 1969). Compared with *Dacus*, however, lateral pouches and the frontal sac do not exist in *Drosophila* (Poulson, 1950). It has been generally accepted that the eye-antennal discs are formed by invagination of the region of ectoderm reserved for eye and antennal development, during the process of head involution (Kankel et al., 1980). The flattened sacs created by the invagination retain an epithelial organization. For each of the sacs, one surface becomes the eye-antennal anlage, and will give rise to the compound eye, while the other surface becomes the peripodial membrane. The size of the disc increases slowly by cell division during the embryonic stage.

B. Larval development

During larval development, the eye-antennal discs enlarge dramatically by further cell division. They are first recognizable at about 16 hours after hatching (Chen, 1929), and are attached to the optic lobe through an optic stalk first visible by light microscopy only at about 24 hours after hatching (Steinberg, 1941). In fact, the optic stalk makes its appearance much earlier (Campos-Ortega and Hartenstein, 1985). The visible separation between the eye and antennal region of the discs starts to occur during the second-instar larva (Trujillo-Cenóz, 1985). Throughout larval development, the discs undergo cellular proliferation. Towards the beginning of the third instar, each eye disc contains about 2,000 cells (Becker, 1957). Pattern formation among these cells begins only at the early third instar, however, when the cells at the posterior of the disc begin to organize into ommatidial clusters (Ready et al., 1976). This organization proceeds as a wave of
development that spreads in an anterior direction, across the eye disc. To distinguish the proliferative activities before and after the start of obvious pattern formation, mitotic activity that occurs before pattern formation has been termed the first mitotic wave, while the second mitotic wave has been used to indicate mitotic activity that occurs after pattern formation starts (Ready et al., 1976)

**Pattern formation.** Pattern formation is initiated at the posterior edge of the eye disc. It then advances to the anterior with the addition of newly organized ommatidial clusters at the front edge of the pattern formed previously (Ready et al., 1976), enlarging the area of patterning. Anterior to the area of patterning is a distinct indentation, called the morphogenetic furrow, which runs across the eye disc. Posterior to the furrow, a gradient of maturity exists amongst the ommatidial clusters: immediately behind the furrow are five-cell clusters; more posteriorly there exist eight-cell clusters (Tomlinson and Ready, 1987). These cells are destined to form the photoreceptors of the ommatidium, and are the first cells to differentiate. $^3$H-thymidine labeling studies of cell division in relation to pattern formation have revealed that the five-cell clusters are formed from the first mitotic wave, and that the five cells comprise the precursor cells of R2, R3, R4, R5 and R8 of the ommatidial cluster, while the remaining three photoreceptors, R1, R6 and R7, are added to the cluster during the second mitotic activity (Ready et al., 1976; Campos-Ortega and Hofbauer, 1977). Neuronal differentiation in an ommatidium, as indicated by the sequence of acquisition of immunoreactivity to neuronal markers, follows the sequence of cellular clustering during ommatidial assembly. Starting with R8, there follows the pairwise expression of neuronal antigens in R2/R5, R3/R4 and R1/R6. R7 is the last photoreceptor to express neuronal antigens (Tomlinson and Ready, 1987). Studies of genetic mosaics have clearly indicated that cells from each ommatidium are not derived from a single mother cell, because at the border of clones there is a zone of mixing of cells of different genotype, with cells of different origins contributing to a single ommatidium (Ready et al., 1976).

From this evidence and from the sequence of assembly of cells into a cluster, it was
correctly inferred that the identity of each photoreceptor is determined not by its lineage (Ready et al., 1976; Lawrence and Green, 1979), but by the positional cues that are encoded by the neighbouring cells with which the photoreceptor comes into contact in the cluster (Tomlinson and Ready, 1987; Banerjee and Zipursky, 1990). Each cell receives cues from the cells to which it adds itself in the cluster, for which the tyrosine kinase-Ras 1 pathway of determination in R7 is an example (Krämer et al., 1991; Dickson, 1995). The non-visual cells in the ommatidia are also generated by the second mitotic wave. These are the primary, secondary, tertiary pigment cells, cone cells and bristle mother cells. Unlike the ommatidium, the mechanosensory bristles of the eye are derived from a single mother cell (Perry, 1968).

C. Pupal development

Early pupal development. Pattern formation continues in eye discs into the first third of pupal development. During that period, cells surrounding the ommatidial cluster generate an interommatidial lattice composed of the three types of pigment cells and the bristle cell complex. The morphogenetic furrow reaches the anterior edge of the eye disc at about 10 hours after pupariation and eversion of the disc occurs shortly thereafter (Cagan and Ready, 1989). Cell-cell interactions again play an important role in determining the cell identity of the pigment cells (Cagan and Ready, 1989). The primary pigment cells are detected at about 22 hours after pupal development. Secondary and tertiary pigment cells are formed after this (Cagan and Ready, 1989; Wolff and Ready, 1993). Complete cellular pattern formation is achieved by mid-pupal life (Wolff and Ready, 1993).

The mechanosensory bristle complex consists of four cells: the tormogen, which secretes the socket; the trichogen, which secretes the bristle; a mechano-sensory neuron; and the thecogen which is a glial cell. Late in pupal life, the tormogen and trichogen degenerate, leaving just two cells in the bristle complex (Cagan and Ready, 1989). The
four cells of the bristle group are generated from a single founder cell which divides twice. Cell divisions generating bristle cells commence at the centre of the eye and spread radially, at about 14 hours after pupariation, in a pattern wholly different from that generating the retina (Cagan and Ready, 1989).

**Late pupal development.** After pattern formation is complete, terminal cellular differentiation of the ommatidium to generate its specialized structures takes place during the final two-thirds of pupal development (Cagan and Ready, 1989; Wolff and Ready, 1993). The rhabdomeres are formed from the apical membrane of the photoreceptor cells. This subsequently extends along the entire axial margin of the photoreceptor. Screening pigments differentiate; lenses and bristles are secreted (Cagan and Ready, 1989). The first appearance of brown ommochrome pigment is detected at 48 hours after pupariation (Shoup, 1966), and it darkens as the pupa develops. The earliest time to detect the red drosopterines is about 90 hours after pupariation (Shoup, 1966). All cells except the bristle complex contain pigment granules (Cagan and Ready, 1989), which contain screening pigments.

**Programmed cell death.** Although limited, cell death also occurs in the larval eye disc (Wolff and Ready, 1993). At the beginning of pupation, the eye disc still contains more cells than it needs to make functional ommatidium. After cells in the interommatidial lattice adopt their individual fates, extra cells are eliminated by a process of programmed cell death (or apoptosis) to generate the regular hexagonal lattice (Cagan and Ready, 1989).

Detailed progress of apoptosis at the ultrastructural level is reviewed by Wolff and Ready (1993).

### 1.2.2. Optic lobe

Each optic lobe consists of three layers of neuropil. From distal to proximal, these neuropils are the lamina, medulla and lobula complex, which is further divided into an anterior lobula and a posterior lobula plate. Although initially formed far from the eyes, in
the adult the lamina lies just beneath the retina. The cell bodies of the chief lamina
interneurons, the monopolar cells, form the cortex of the lamina beneath the basement
membrane. Proximal to the lamina cortex is the lamina neuropil proper, arranged as
columns of receptor terminals and their columnar lamina interneurons, each column
collectively called a cartridge. The cellular organization of the medulla is similar to that of
the lamina, in that the cell bodies are located in a distal cortex overlying a columnar neuropil
located proximally. Between the lamina and the medulla exists the fibre tract of the first
optic chiasma. The neuropils of the lobula complex also contain the neurites of cells
arranged in a columnar array, with the second optic chiasma connecting between the
proximal face of the medulla neuropil and the inner faces of the lobula and the lobula plate
(Braitenberg, 1970; Strausfeld and Nässel, 1980).

Compared with the other parts of the central nervous system (CNS), the optic lobes
have one of the most visibly highly-ordered anatomical organizations. The relay of visual
information from the compound eye to each successive station of the optic neuropils is
scrupulously retinotopic (Braitenberg, 1970; Meinertzhagen, 1976). Photoreceptors R1-
R6 have short axons which terminate in the lamina, where they provide synaptic inputs
upon lamina cells, especially three of the monopolar cells (L1-L3) and amacrine cells of the
cartridges (Meinertzhagen and O'Neil, 1991). Within a single ommatidium, the optical
axes of each of R1-R6 differs slightly from the others. On the other hand, each of R1-R6
within a single ommatidium views the same point in space (i.e. shares the same visual axis)
as a neighbouring photoreceptor in each of five other adjoining ommatidia (Kirschfeld,
1967). These are R1 in one ommatidium, R2 in another, R3 in a third etc. To achieve
functional retinotopy, each photoreceptor of an ommatidium projects to a different cartridge
in the underlying lamina, where its terminal joins those of the five other photoreceptors that
view the same point in space, to achieve neural superposition (Braitenberg, 1967). As a
result, each cartridge receives a projection from exactly those six photoreceptors, one each
of R1-R6 from six neighboring ommatidia, that share the same optical axis. The number of
lamina cartridges is thus the same as that of the overlying ommatidia (Strausfeld and Nässel, 1980). The photoreceptors R7 and R8 have long visual fibres, which by contrast, penetrate the lamina and terminate in the medulla (Melamed and Trujillo-Cenóz, 1969; Fischbach and Dittrich, 1989). The orientation of the ommatidial retinotopic map in the retina and lamina is the same, whereas it is reversed about a vertical plane in the medulla, as the result of the optic chiasma (Horridge and Meinertzhagen, 1970).

Development of the optic lobes

In holometabolous insects, such as Drosophila, the proliferative activity of neuroblasts during embryogenesis forms only the larval central nervous system (Campos-Ortega and Hartenstein, 1985). The adult nervous system is produced instead by postembryonic neurogenesis, during which imaginal neurons arise either from sets of progenitor cells that are already present in the embryo, set aside for later development (Campos-Ortega and Hartenstein, 1985), or by the reorganization of some larval elements together with the histolysis of others (White and Kankel, 1978; Truman, 1990). Although the optic lobe is the product of postembryonic neurogenesis, both embryonic and postembryonic development thus play important roles in its formation (Tix et al., 1989; Meinertzhagen, 1973). An recently updated description of optic lobe development in Drosophila is provided by Meinertzhagen and Hanson (1993).

A. Embryonic development

Embryonic development of the optic lobe is closely associated with the initial formation of the larval eyes (Bolwig's organs). Precursors for both Bolwig's organ and the primordium of the optic lobe originate in the dorsolateral region of the embryonic head (Hartenstein and Campos-Ortega, 1985; Green et al., 1993), which appears as a deep groove dorsocaudally in the procephalic lobe, the posterior procephalic region (Green et al., 1993; Meinertzhagen and Hanson, 1993). The embryonic generation of the CNS
occurs by delamination of dispersed subsets of stem cells, neuroblasts, from the neuroectoderm. The optic lobes and Bolwig's organ, on the other hand, together form by the invagination of a placode of densely packed cells from the ectoderm of the posterior procephalic region. During the process of invagination, the cells that will form Bolwig's organ segregate from the ventral lip of the invagination, remaining in the head epidermis. The remainder of the optic placode invaginates and seals up as an epithelial vesicle, losing contact with the head epidermis and attaching to the lateral surface of the developing supraesophageal ganglion (Green et al., 1993). The cells of the optic lobe vesicle remain mitotically inactive until the animal reaches larval life (White and Kankel, 1978), at which point they rapidly regain mitotic activity (Hofbauer and Campos-Ortega, 1990).

B. Postembryonic development

At the time of hatching from the egg, the first-instar larval CNS consists of two brain hemispheres, or supraesophageal ganglia, and the ventral nerve cord. Both the brain hemispheres and the ventral nerve cord are composed of an external cellular cortex and an internal neuropil. The primordium of the optic lobe, which originally consists of 30-40 precursor cells, is located postero-laterally in the cell body layer of each brain hemisphere (Hofbauer and Campos-Ortega, 1990). These cells soon become neuroblasts and commence mitosis. Metamorphosis of the optic lobe vesicle during the early larval stages gives rise to the optic anlage. Subsequent proliferation and segregation of the neuroblasts in the anlage produces two centres, the outer and inner optic anlagen, which are first recognizable during the second half of the first larval instar (Hofbauer and Campos-Ortega, 1990; Meinertzhagen and Hanson, 1993). The two anlagen are closely located, with the outer optic anlage lying superficially, surrounding the entrance of the optic stalk, while the inner optic anlage resides deeply in the brain hemisphere, wrapping around the fibres of the optic stalk (Meinertzhagen and Hanson, 1993). Lamina and distal medulla are produced by the lamina-forming limb and medulla-forming limb of the outer optic anlage, respectively.
These two limbs are separated by a narrow indentation on the third-instar optic anlage (Meinertzhagen and Hanson, 1993). The proximal medulla and the lobula complex derive from the inner optic anlage (Meinertzhagen and Hanson, 1993). Different cell division patterns are present during the optic lobe development. Initially neuroblast numbers increase from symmetrical cell divisions. By asymmetrical divisions, each neuroblast divides in stem-cell mode to produce one neuroblast and one ganglion mother cell, which in turn divides to produce two ganglion cells through a symmetrical cell division (Hofbauer and Campos-Ortega, 1990).

C. Dependence of the optic lobe cells on innervation from the retina

The innervation of the optic lobe precursor cells (the neuroblasts and their progeny, ganglion mother cells) by retinal axons is crucial for the normal development of the adult optic lobes (Meinertzhagen, 1973; Fischbach and Technau, 1984; Selleck and Steller, 1991; Selleck et al., 1992). Nevertheless, early morphogenetic changes in the developing first-instar optic lobe are independent of the development in the eye-disc, even though the attachment of the eye-discs to the optic lobes through the optic stalk is established as early as 24 hours after hatching (Steinberg, 1941). This independence can be reasoned from the simple fact that photoreceptor differentiation only starts to occur during the third-instar larva. During the mid-third instar, however, extensive morphogenesis begins to take place in the optic lobe in conjunction with the morphogenesis undergoing concurrently in the eye discs. As pattern formation in the eye disc proceeds from the posterior to the anterior edge of the disc, in the wake of the morphogenetic furrow (Ready et al., 1976), corresponding bundles of photoreceptor axons arising from the developing wavefront of assembling ommatidia extend and reach the developing optic lobe through the optic stalk. This innervation is important for the formation of the adult optic lobe (Meinertzhagen, 1973; Meinertzhagen and Hanson, 1993) in two ways that are documented for the lamina. The sequence of cell birthdates in both lamina and medulla mirrors the order of the pattern
formation of the retina, the cells in these two cortices being generated at approximately the
time at which retinal axons contact them (Kankel et al., 1980; Hofbauer and Campos-
Ortega, 1990). In the lamina cortex, there is evidence for direct regulation of the cell cycle
by ingrowing retinal axons (Selleck and Steller, 1991; Selleck et al., 1992). The axons
regulate the G1-S transition in postembryonic lamina precursor cells, so as to match the
number of lamina cartridges that become established to the number of ingrowing
photoreceptor axon bundles that innervate them. A second interaction identified in the
lamina, possibly signaled by the same ingrowth of receptor axons but occurring with
lamina cells that are further along the lamina anlage and therefore older, is to trigger
differentiation and axonogenesis (Meinertzhagen and Hanson, 1993). Mutational studies
have clearly demonstrated the dependence of optic lobe development, especially the lamina,
on photoreceptor innervation (Meyerowitz and Kankel, 1978; Fischbach, 1983). The
survival and differentiation of columnar neurons in the medulla is not so crucially
dependent on the innervation from photoreceptors, because at least some cells in the
medulla cortex do not behave as their counterparts in the larva and form normally even in
congenitally eyeless flies (Fischbach, 1983). Although innervation from photoreceptors is
critical for the initial development of the adult optic lobes (Meinertzhagen, 1973;
Meyerowitz and Kankel, 1978; Selleck and Steller, 1991), ablation of photoreceptors by
the conditional expression of a toxin gene suggests that their innervation is not required
continuously for the maintenance of the optic lobe after mid-pupal stages (Kunes and

D. Dependence of photoreceptor cells on interaction with optic lobe

By contrast to the lamina, the compound eye is developmentally autonomous. This
was suggested long ago by transplantation experiments (Bodenstein, 1939; 1941) and a
variety of other evidence (Schneider, 1964; Wolski and Wolski, 1971; Anderson, 1978;
Marcy and Stark, 1985). The long-term survival of the adult retina does however depend
on an interaction with its optic lobe (Campos et al., 1992). Even though many examples elsewhere in the nervous system have shown the retrograde degeneration of certain neurons after loss of their target cells, a possible retrograde trophic influence from the optic lobe to the photoreceptor cells has only recently been suggested (Campos et al., 1992). In the mutant flies disconnected (disco), the photoreceptors form normally but their axons fail to reach their targets, in the optic lobe (Steller et al., 1987). Flies carrying the disco mutation exhibit degeneration of their photoreceptors two weeks after eclosion (Campos et al., 1992). This observation suggests that a possible retrograde trophic influence from the optic lobe is required for maintenance of the adult retina beyond two weeks (Campos et al., 1992). Whether the retrograde influence is because of a trophic factor, or whether this might arise from the optic lobe neurons or from glial cells in the optic lobe is not known, however (Campos et al., 1992).

II. Hormonal regulation of development

It has long been realized that the development of insects is regulated by hormonal signals (Kopeć, 1922; Wigglesworth, 1934; Fraenkel, 1935; Bodenstein, 1942). The metamorphosis occurring during postembryonic development in holometabolous insects, which gives rise to the adult visual system as well as other adult structures, in particular, is under precise endocrine regulation. Four hormones have been shown to be important during the process of moulting and metamorphosis. These are the prothoracicotropic hormone (PTTH), the ecdysteroids, juvenile hormone (JH) and the eclosion hormone (EH). Among the four hormones, the ecdysteroids have received extensive attention, because of their clearly identifiable roles in inducing moulting and metamorphosis in insects. The entire endocrine system, however, works in concert to achieve the coordinated development of the organism.
The endocrine systems of different insects share many similarities. The hormones regulating insect development are, for example, not species-specific. It has been long known that a hormone (Wigglesworth, 1936; Bodenstein, 1942) or endocrine organ (Bodenstein, 1942) responsible for metamorphosis in one species can also induce metamorphosis in another species (Bodenstein, 1942). The general anatomy and physiology of the endocrine system is also similar among insects. Therefore, knowledge of hormone action in one insect can potentially be applied to another. Indeed, most of our knowledge of the hormonal regulation of development in Drosophila is derived from knowledge of the action of hormones in other insects. Only recently has the advantage of Drosophila genetics been used to elucidate the mechanism of hormonal action at the molecular level, in particular with the identification of the ecdysone-responsive genes (Natzle et al., 1992; Stone and Thummel, 1993; Karim et al., 1993) and the cloning of the ecdysteroid receptor (Koelle et al., 1992). A comprehensive summary of hormonal function during Drosophila development has been given by Riddiford (1993).

1.3.1. Historical background

The hormonal control of insect development was first demonstrated by Kopeč in the gypsy moth, Lymantria dispar (Kopeč, 1917; 1922). In his studies on the process of metamorphosis in this insect, Kopeč ligatured a final instar larva in the middle of the body. If the ligature were applied sufficiently early, only the anterior part of the body transformed to the pupa, with the posterior part of the body failing to undergo metamorphosis. Late application of the ligature, however, did not prevent metamorphosis of the posterior part. From these results he concluded that during a critical period of development a hormone released from the brain is essential for metamorphosis to occur. Despite the inaccuracy of localising the source of the hormone in the anterior of the larva, Kopeč's discovery was the first evidence in any animal that the nervous system could also function as an endocrine
gland (Kopeć, 1922). Not until much later did Scharrer (1928) demonstrate that certain hypothalamic neurons of the vertebrate brain have an endocrine function.

The classical experiments of Wigglesworth (1934) on the postembryonic development of the bloodsucking bug *Rhodnius prolixus*, set the foundation for insect endocrinology. In *Rhodnius*, moulting normally occurs after the intake of a blood meal. Wigglesworth found that moulting after a blood meal was arrested by decapitation prior to a critical period. He also found that a young larva decapitated before the critical period could moult provided it receiving a transfusion of haemolymph from an older larva. Wigglesworth's experiments thus not only confirmed Kopeć's findings, but also provided evidence that the moult-inducing factor released by the head was present in the haemolymph.

The requirement for endocrine organs in the thorax to allow moulting to occur was indicated by several experiments from the same period. Hadorn and Neel (1938) demonstrated that extirpation of ring glands (see section 1.3.2) from the thorax prevents larvae from pupariation in the blowfly, *Calliphora erythrocephala*, and in *Drosophila*, while Burtt (1938) successfully induced metamorphosis in a young larva by implanting ring glands from old larvae. By transplanting imaginal discs and ring glands, Bodenstein (1943) further indicated that the ring gland was not only important in inducing metamorphosis, but also responsible for further imaginal differentiation. The prothoracic glands were identified as the source of a hormonal factor in the thorax that controls moulting, in the moths *Bombyx mori* (Fukuda, 1940) and *Hyalophora cecropia* (Williams, 1948).

Although the hormonal factors released by both the brain and the prothoracic gland were essential for moulting and metamorphosis, the next step was to identify that the secretion of the prothoracic gland is, in turn, stimulated by the liberation of the brain hormone (Piepho, 1942; Williams, 1947; 1952). Plagge (1938) noticed that the brain could cause pupariation in debrained *Deilephila* larvae but could not induce pupariation in
the posterior part of a ligated larva. Williams (1947) performed similar studies by
implanting the brain into isolated sections of debrained pupae, and then following
subsequent development. He found that only the anterior half of a pupa developed into an
adult when implanted with a brain, while the posterior half remained pupal. The posterior
half could develop to an adult only if both brain and prothoracic glands were implanted
(Williams 1947). Thus hormone released from the brain controls the function of the
prothoracic gland. The brain hormone responsible for moulting is now termed
prothoracicotropic hormone (PTTH), for its stimulating activity on the prothoracic gland,
and the hormone released by the prothoracic gland is named moulting hormone, or
ecdysone, one of a family of ecdysteroids.

Another hormone present in the haemolymph of young larvae, the juvenile hormone
(JH), also controls moulting. Wigglesworth (1934, 1936) demonstrated that a hormonal
factor released from the head was able to inhibit metamorphosis. By cutting through the
head at different levels, he located the source of the hormone in the corpus allatum of the
brain. Transplanting corpora allata from young larvae into last-instar larvae could induce
an extra moult, producing giant larvae. Adult *Rhodnius* could also be made to undergo an
additional moult in the presence of abundant hormone from the corpora allata
(Wigglesworth, 1940). Removing the corpus allatum from young larvae, on the other
hand, gave rise to a miniature adult in the silkworm, which underwent premature
metamorphosis (Bounhiol, 1938). The hormone released from the corpus allatum was
therefore named juvenile hormone.

The existence of a fourth hormone, eclosion hormone (EH), was realized much later
than the above three hormones. As the name implies, this hormone is important in
initiating eclosion behaviour. The first indications of EH came from studies of eclosion
behaviour in giant silkmoths (Truman and Riddiford, 1970). Normal eclosion occurs only
at a certain time of day. When the brain was removed early in adult development, eclosion
occurs randomly throughout the day and night. This abnormality of eclosion could be
corrected if the removed brain were re-implanted at the tip of abdomen of the pupa, creating a so-called "loose-brain" (Truman and Riddiford, 1970). Homogenates from brain and corpora cardiaca could trigger eclosion at any time of the day in pharate moths (Truman and Riddiford, 1970; Truman, 1971). The release of EH from the corpora cardiaca was then soon confirmed in *Antheraea pernyi* moths (Truman, 1973). EH is neither species- nor genus-specific, as indicated by the effects of transplanting brains between *Cecropia* and *Antheraea* (Truman and Riddiford, 1970).

1.3.2. The components of the endocrine system

The endocrine system of insects is classically divided into four parts: the neurosecretory cells in the protocerebrum, the corpora cardiaca, the prothoracic gland (according to their locations, also known in different insects as "ventral glands", "intersegmental", "peritracheal" or "pericardial glands"), and the corpora allata. In *Drosophila*, the corpora cardiaca, the prothoracic gland and the corpora allatum together form the ring gland of the larval brain.

A. Protocerebrum

The neurosecretory cells of the protocerebrum secrete the prothoracicotrophic hormone, PTTH. The location of these neurosecretory cells was first suggested to lie in the dorsal region of the brain in *Rhodnius*, because of the histological appearance of secretory granules in certain cells in that area (Hanström, 1938). Using tissues from different parts of the brain to examine their ability to elicit moulting in decapitated larvae, Wigglesworth (1940) was able to confirm that the location of these cells was the pars intercerebralis of the protocerebrum. There are four groups of neurosecretory cells in the insect pars intercerebralis: two medial and two lateral groups (Richards, 1981). The lateral cells are the source of the PTTH that stimulates the production and secretion of ecdysone from the prothoracic gland (Bollendacher et al., 1979) for normal larval development.
(Gibbs and Riddiford, 1977). A single neurosecretory cell amongst the lateral cells was found to be the source of PTTH in the pupal brain of *Manduca sexta* (Agui et al., 1979). The medial cells, on the other hand, control the synthesis of ecdysone in adult female ovaries (Charlet et al., 1979; Richards, 1981).

Although PTTH was the first hormone discovered in insects, and was the first neurohormone discovered in any animal, its chemical structure and therefore the mechanism of its action are still the least understood of the hormones that control insect development. It is now generally accepted that PTTH is a peptide (Ishizaki et al., 1977; Bollenbacher and Granger, 1985), among many possible molecules or classes previously suggested (Kirimura et al., 1962; Williams, 1967). The molecular weight of the hormone varies greatly from assay to assay, and from species to species (Bollenbacher and Granger, 1985), ranging from 4 kd (Susuki, 1982) to 25 kd (Kobayashi and Yamazaki, 1966; Kingan, 1981). This wide range of molecular weight possibly suggests the existence of more than one PTTH peptide (Bollenbacher and Bowen, 1983) or a preprohormone. With so little knowledge about its chemical nature, the mechanism by which PTTH exert its function on the prothoracic glands is also not yet known. Nevertheless, the ability of cAMP to stimulate the production and secretion of ecdysone in the prothoracic gland has indirectly suggested that, upon binding to its receptor at the target cell, PTTH might use cAMP as a second-messenger (Vedeckis et al., 1976) to regulate the production of ecdysone from the prothoracic gland.

**B. The ring gland**

The ring gland is situated dorsally between the two brain hemispheres in the fly larva. The lateral extremities of the gland encircle the aorta like a ring; hence the name ring gland. Each of the three separate endocrine organs of the ring gland produce and/or store different hormones. The corpus allatum produces juvenile hormone (Wigglesworth, 1940). It also serves as a neurohaemal organ by means of which PTTH is stored and
released into the haemolymph (Agui et al., 1979). The prothoracic gland produces and secretes ecdysteroids, while the corpora cardiaca contains eclosion hormone (Truman, 1971).

a) Juvenile hormone

Juvenile hormone is synthesized in the corpora allata (Wigglesworth, 1940). Four members have been identified in the JH family: JHI (Röller et al., 1967), JHII (Meyer et al., 1968), JHIII (Judy et al., 1973) and JHO (Bergot et al., 1980). All of them are fatty acid derivatives, with a sesquiterpenoid skeleton. Upon synthesis, JH is immediately released into the haemolymph, and is not stored (Feyereisen, 1985). The function of JH is to balance the effect of the ecdysteroids, and thus to prevent metamorphosis during larval moults (Riddiford, 1985), and to prevent adult differentiation during the pupal moult (Kiguchi and Riddiford, 1978). The activity of the corpora allata stops prior to pupal ecdysis, resulting in a decline in JH, and the initiation of metamorphosis under the influence of ecdysteroids (Riddiford, 1993).

b) Ecdysteroids

The terms ecdysone and ecdysteroid have both been used as generic terms for insect moulting hormones. Over 20 chemically different hormones in this family have in fact so far been identified. To avoid confusion, the term ecdysteroid is used in a generic sense, while ecdysone is used here to refer to only one of these ecdysteroids. As the name ecdysteroid implies, the moulting hormones are steroids. Among the large number of naturally occurring ecdysteroids, ecdysone (previously named α-ecdysone) and 20-hydroxyecdysone, or 20-HE (previously named β-ecdysone), are the predominant ecdysteroids found in extracts of insects (Butenandt and Karlson, 1954; Karlson, 1956). The prothoracic glands are thought to be the major source of ecdysone during most of postembryonic development. After release from the prothoracic gland, ecdysone is converted to 20-HE in the periphery by the fat body (King, 1972), possibly through cytochrome P-450-dependent mono-oxygenase systems (Smith, 1985). Although
ecdysone and 20-HE elicit similar effects \textit{in vitro}, studies have indicated that 20-HE is much more potent than is ecdysone (King, 1972; Milner and Sang, 1974). It is now generally accepted that 20-HE is the active form of insect ecdysteroid, and that many of the ecdysone effects observed earlier have been due to its conversion \textit{in vivo} to 20-HE (Smith, 1985). Like vertebrate steroid hormones, ecdysteroids exert their effects by binding to steroid receptors (Yund et al., 1978). Affinity studies confirm the bio-activity of ecdysteroids (Yund and Osterbur, 1985), indicating that 20-HE has greater affinity for the receptor than does ecdysone.

The differentiation of imaginal discs is regulated by the presence of ecdysteroids during metamorphosis. When eye-antennal discs are cultured in a medium containing 0.2\(\mu\)g/ml of 20-HE, they fuse \textit{in vitro} to produce the adult pattern of cuticular structures between the two eyes (Milner and Haynie, 1979). Yund (1980) concluded from a review of \textit{in vitro} studies on imaginal disc differentiation that imaginal discs are primed during the late larva to undergo morphogenesis, awaiting only the ecdysteroid stimulus.

The synthesis and secretion of ecdysone by the prothoracic glands is regulated by PTTH, as previously mentioned. The prothoracic glands degenerate after metamorphosis and the ecdysteroids detected in many adult female insects are produced and secreted not by the prothoracic glands but by their ovaries (Hagedorn et al., 1975; Hagedorn, 1985), where their synthesis is regulated by the medial cells of the pars intercerebralis (Richards, 1981).

c) Eclosion hormone

The corpora cardiaca release eclosion hormone, EH (Truman, 1971). The hormone is not produced in the corpora cardiaca, however. Two discrete regions in the CNS have been suggested to be the source of EH (Truman, 1985). These are the neurosecretory cells in the ventromedial part of the brain, and those in the ventral ganglia. Eclosion hormone in the corpora cardiaca comes from neurosecretory cells in the brain which send their axon terminals to the corpora cardiaca, and only from here is EH released into the haemolymph.
Removal of the brain early in adult development diminishes the concentration of EH in the corpora cardiaca of pharate adult *Manduca* (Truman, 1985). The concentration of EH is high in the brain and corpora cardiaca (Truman, 1973), although EH has also been detected in other regions of the nervous system (Taghert et al., 1980). Eclosion hormone extracted from pharate adult *Manduca* as well as pharate pupae has been characterized, and suggested to be a peptide of molecular weight 8.0-8.5 kd (Reynolds and Truman, 1980; Taghert et al., 1980). The release of EH is under circadian control, restricting ecdysis to a specific time of day, usually between one and three hours after EH release (Riddiford, 1993).

1.3.3. Endocrine regulation of insect development

The co-operation of these endocrine systems is important for the normal development of an insect. The endocrine regulation of insect development can be summarized as follows: (i) The balance between ecdysteroids and JH is important in determining the nature of early development of insects up to the pupal moult. (ii) The surge of ecdysteroids in the absence of JH just after pupariation is necessary to initiate metamorphosis. During metamorphosis, imaginal discs differentiate into adult structures under the influence of ecdysteroids. (iii) The decline in ecdysteroid levels then renders moulting insects sensitive to EH (Truman, 1985), which leads to ecdysis. Sensitivity to EH is not only essential in causing ecdysis behavior, but is also suggested in initiating certain programmed cell deaths that occur after ecdysis (Kimura and Truman, 1990).

III. Tissue culture

1.4. Tissue culture as a means to study development of the nervous system

The profound complexity of the nervous systems, with its heterogeneous cell types and elaborate organization, has provoked many attempts to study the fundamental
mechanisms involved in both its function and development by using simpler model systems. A variety of in vitro models, more manageable for experimentation than the nervous system in vivo, have been developed to serve this purpose. Selection of an optimal in vitro model depends, however, on the particular problem under investigation. Freshly isolated mature nervous tissue can be maintained in physiological solutions, but only for short periods (several hours). This in vitro method has been widely used in electrophysiological studies of the nervous system, because complex synaptic functions are maintained in such isolated tissues. For long-term maintenance of nervous tissues in vitro, however, tissue culture is necessary. Tissue culture techniques not only allow direct microscopic observation of nervous tissue over long periods, but also permit immature nervous tissues to exhibit the sequential development of characteristic structures and functions in vitro.

The pioneer studies of nervous tissue in culture were those of Harrison (1907, 1910) soon after the turn of the century. Using the neural tube from frog larvae in culture, Harrison provided direct evidence that nerve fibres were outgrowths of nerve cell bodies, and carefully documented the behaviour of growth cones in culture (Harrison, 1910). Since then, tissue culture techniques have greatly advanced and have made possible major contributions to our understanding of various aspects of nervous system development and function.

Tissue culture techniques not only serve as a means to investigate properties of cells and tissues in a simplified environment, but the search for conditions to maintain and to support the development of cells and tissues in vitro also makes the techniques themselves objects of investigation. A wide range of culture methods have been developed, each designed to answer specific questions.

Progress in the development of tissue culture methods for vertebrate nervous systems is far more advanced than that for insect nervous systems, so that methods for vertebrate tissue/cell culture have now become highly standardized. As a result, it is
feasible to obtain standard culture media, other reagents and culture equipment commercially to establish vertebrate tissue cultures. The development of insect tissue culture techniques still lags behind, and in many ways imitates the tissue culture methods used in vertebrates, and as a result is still at a very phenomenological stage. The general culture methods used in vertebrate studies will therefore be presented first, followed by a discussion of insect tissue culture systems.

1.4.1. General strategies used in the culture of vertebrate neural tissue

A. Organ culture. The earliest attempts at neural culture were made on intact fragments of nervous tissue (Harrison, 1907, 1910). This kind of culture contains heterogeneous cell types derived from the tissue of origin. The procedure for organ culture involves explantation of small fragments from the central nervous system, which are then dissected manually or sliced by a mechanical tissue sectioner, onto culture substrates. According to the pattern of growth in culture, Maximow coined the term "histotypic" to describe the diffuse migration of cells from a cut surface, and the word "organotypic" to describe cultures which develop and differentiate while still preserving the general cellular architecture of the original tissue (Crain, 1976). Organotypic cultures are widely used in studies of the vertebrate nervous system, because the retention of intercellular relations among the different populations of cells in the tissue permits close to normal development in vitro. As a result, this method can be used to observe the development of a living tissue in a way that is not possible in vivo. Normal synaptic connections are maintained or formed in organotypically cultured nervous tissue, and this provides access to them for experimental manipulation.

Organotypic cultures can be prepared in a number of ways (Gähwiler, 1988). The most commonly used methods make use of the Maximov assembly (Murray, 1971; Dreyfus and Black, 1990), roller-tube techniques (Gähwiler, 1981; 1984) and the interface-culture (Stoppini et al., 1991). The Maximow assembly makes use of a depression slide
and a glass coverslip. After culture, the coverslip is sealed on top of the depression slide by wax. This method is laborious to prepare but has the advantage of that cultures can be viewed with the microscope throughout the culture period, without having to disassemble the culture. Cultured tissues are highly differentiated and remain several cell layers thick, so that they are ideal for studies requiring the preservation of three-dimensional intercellular relationships. Organotypic cultures prepared with this method can usually be kept for months (Toran-Allerand, 1990). The roller-tube method was firstly introduced by Hogue (1947). After several modifications (Costero and Pomerat, 1951; Schlämpfer et al., 1972) and detailed characterization by Gähwiler (1981; 1984), the roller-tube method has become a popular technique for organotypic culture. Organotypic cultures prepared by this method also retain a high degree of neuronal differentiation. One important characteristic of this technique is that the cultured tissues gradually thin out to become a monolayer, or nearly so, over a period of 2-3 weeks, making it finally possible to visualize the morphology of individual cells using phase contrast microscopy (Gähwiler, 1981), important for the electrophysiological study of morphologically identified neurons. Observation of the culture requires the removal of cultures from the roller tube, however. Recently developed interface-culture techniques (Stoppini et al., 1991) have quickly gained in popularity, because of their simplicity of preparation. Cultures are established on the membranous surface of a tissue-culture insert which sits in a culture well. Culture medium is added just sufficient to reach the surface of the implanted tissue. As for the Maximow assembly, the nerve tissues in the interface culture vessel can be observed throughout the culture period and they remain several cell layers thick (Stoppini et al., 1991). Long-term survival (up to 6 weeks) of nervous tissue is possible in culture, at least for hippocampal slices (Stoppini et al., 1991). Organotypically cultured brain slices using these three techniques all exhibit morphological and electrophysiological characteristics similar to the in vivo tissues from which the slices were taken (Crain, 1976; Zimmer and Gähwiler, 1984; Yamamoto et al., 1992). Tissues from adjacent or remote areas of the CNS can be co-cultured with
organotypic methods. These co-cultures are invaluable in studies of the projections and transmission between the two co-cultured brain areas (Zimmer and Gähwiler, 1984; Yamamoto et al., 1992; Frotscher and Heimrich, 1993).

B. Dissociated primary cell cultures. Techniques have also been developed to dissociate nervous tissues into cell suspensions and to culture them as dissociated neuronal cultures. Dissociated cell cultures provide a high resolution view of the nervous tissue of interest at both the cellular and molecular level, one which is not attainable with organ cultures. This method provides maximal access to the microscopic appearance of cellular morphology and permits direct manipulation of neurons and their synaptic connections. Dissociation is usually achieved by mechanical procedures or by various enzymatic treatments, or with a combination of both.

The complexity of the dissociated cell cultures ranges from highly purified populations of cells to mixtures of heterogeneous populations. Selection of the complexity of cell culture, again, depends on the questions to be answered. Highly purified cell cultures are very useful in pharmacological studies under controlled conditions, while co-cultures of different cell populations are used to study the specific interactions of defined cell populations.

In order to obtain enriched or even purified cell populations from the nervous system, a number of techniques have been developed. These techniques are generally based on the differences of individual cell types in either their nutritional requirements or their adhesion properties. Okun obtained relatively "pure" chicken embryo dorsal root ganglion neurons by adhering the non-neuronal cells to glass beads before plating the neurons (Okun et al., 1972). In vertebrates, neurons differentiate from neuroblasts, which have already undergone terminal postmitotic differentiation. Taking advantage of this, antimitotic agents such as cytosine arabinoside or aminopterine can be applied to the culture to inhibit the mitotic activity of non-neuronal cells from nervous tissue. Cultures obtained
in this way contain a relatively low proportion of non-neuronal cells. Virtually pure neuronal cultures can be achieved by using serum-free medium (Bottenstein and Sato, 1979; Skaper et al., 1979; Aizenman and de Vellis, 1987). The rationale behind this procedure is that glia require serum to divide and adhere to substrata. As a result, the serum-free medium selects for the survival of neurons. Different types of neurons can also be separated into subpopulations by means of velocity sedimentation, which segregates cells according to their different sizes (Barkely et al., 1973).

Dissociated cells can be cultured in several different ways, each to answer different questions. Cell suspensions containing all types of cells from a neural tissue can be cultured in bottles mounted on a rotating-shaker apparatus to form three-dimensional aggregates (Moscona, 1961; 1965). This system has been mostly used to study cell-cell interactions amongst heterogeneous cell populations in the central nervous system. Dissociated cells cultured in this way can reaggregate in a systematic way to form organotypic arrays which mimic the condition in vivo, and sometime reconstruct brain structures in vitro (Garber and Moscona, 1972). Detailed procedures for rotating cultures have been described by Goldsmith and Berens (1990). In most cases, however, dissociated cells are cultured as monolayers on different substrates in stationary vessels.

C. Culture of cell lines. Cells from the nervous system can be "immortalized" to generate cell lines. Cell lines have both the properties of tumoral cells, because they are continually mitotic, and the properties of the differentiated cells of the original tissues. Cultures obtained from a single cell line are usually highly homogeneous. Cell lines also provide the advantage of being easy to keep as stocks and thus of yielding large quantities. With the help of molecular biological techniques, cells can be "switched" between tumoral cells and differentiated neuronal cells under a promoter that responds to the change of the external environment. For example, a temperature-sensitive promoter can be used for this purpose. The ready abundance of cell lines means that they have been used in many
bioassays. For example, the PC12 cell line has been used to study neuronal differentiation in response to trophic factors, such as nerve growth factor or NGF (Ferrari et al., 1983).

1.4.2. History of insect tissue/cell culture methods

The history of insect tissue culture is nearly as long as that of vertebrate tissue culture. While vertebrate tissue culture systems have become very well established, however, the development of methods for culturing insect tissue is still in a primitive state. The delay in establishing successful methods for insect tissue culture is largely attributed to the fact that pioneer work on insect tissue culture lacked knowledge of the nutritional requirements that favoured the growth of insect tissues in vitro. Tremendous progress in insect tissue culture has been made during the last few decades, as knowledge of the physiology and biochemistry of insects has increased (Buck, 1953; Wyatt, 1961; Begg and Cruickshank, 1963). Nevertheless the techniques and the culture media employed by each investigator still vary greatly. A number of important reviews in the last decade or so now provide us with a general picture of the development of successful insect tissue culture techniques (Marks, 1980; Beadle and Hicks, 1985). Techniques to establish tissue cultures from a variety of invertebrates, including insects, have been published by Vago (Vago, 1971, 1972), and their growing popularity is evident from regular conferences on this subject (Rehacek et al., 1973; Kurstak and Maramorosch, 1976; Kurstak et al., 1980).

A. The culture media

A major concern in obtaining successful insect tissue culture is the nature of media which bathe the tissue or cells. A suitable medium will provide an environment in which the cellular metabolic activities of the cultured tissue approximates those in vivo. To design such media was, and still is, the main task of insect tissue culture. The composition of that medium need not, for this purpose, be identical to that of the insect haemolymph. Haemolymph does not directly bathe the cells of the insect CNS in vitro, and the various
selective filters offered by the insect's blood brain barrier mean that the composition of the extracellular fluid (the physiological solution that a culture medium aims to replicate) may be related only loosely to the composition of haemolymph.

The initiation of insect tissue culture began soon after Harrison's studies (1907, 1910). Working in the Harrison laboratory, Goldschmidt (1915) was the first to attempt to adapt Harrison's method of tissue culture for insect tissues. Using Harrison's "hanging drop" method and a medium consisting of haemolymph and a simple salt solution, Goldschmidt cultured the testicular follicle cells of *Samia cecropia* and observed maturation of the cells *in vitro*, in a manner analogous to their maturation *in vivo* (Goldschmidt 1915). In spite of this preliminary success, the technique proved unsuccessful in other studies on insects. The difficulties of early attempts were mainly due to two major problems encountered during the culture period. The first problem was the difficulty of maintaining sterility. Elaborate precautions were taken to ensure sterility, but most cultures were still contaminated with bacteria shortly after initiating culture. Nevertheless, for most insect tissues, this problem could be overcome by thorough washing. The second and most important problem was the lack of knowledge of the composition of insect body fluids. In principle, the optimum environment of the growth of most tissues *in vitro* should be close to the composition of haemolymph (Jones, 1962), so that understanding the composition of haemolymph provided valuable guidance in formulating the first culture media.

Vertebrate tissue culture media were used to guide the composition of early culture media for insect tissues. Twenty years after Goldschmidt's culture experiment, Trager (1935) successfully obtained the first observation of mitosis in cells other than germ cells cultured *in vitro*. Later, he succeeded in maintaining the imaginal discs, ovaries and mid-gut of the mosquito for up to 47 days (Trager, 1938). The medium he used in these two studies supposedly contained components favouring the growth of vertebrate tissues *in vitro*. Trager's success suggested the possibilities of extending the use of vertebrate tissue culture media to culture insect tissues. The lack of information on the composition of insect
haemolymph and the success of Trager's experiments impelled early work on designing insect culture media to be based on the knowledge of nutritional requirements for vertebrate tissues in vitro. It is indeed not unreasonable to think that the culture media suitable for vertebrates would also be suitable to meet the nutritional requirement of insect tissues, because the chemical composition as well as the fundamental metabolic pathways used by vertebrate and insect cells are so similar. The media so designed did support short-term survival of nervous tissue from the mosquito (Pfeiffer, 1937) and imaginal discs of Drosophila (Gottschewski and Fischer, 1939). Unfortunately, no significant cell division was evident, a phenomenon that interested most insect tissue culture workers of that time, because insect cell cultures were needed as a vehicle to study virus propagation. The reason for the lack of mitotic activity amongst insect cells when these were held in vertebrate tissue culture media was largely resolved when the physical and chemical properties of insect haemolymph became known (Buck, 1953; Wyatt, 1961).

The composition of insect haemolymph is different from vertebrate fluids in several important aspects. The presence of organic phosphates in haemolymph (Wyatt, 1961) and of the non-reducing disaccharide trehalose as the principle sugar in haemolymph (Wyatt and Kalf, 1957) are two examples of differences between vertebrate and insect blood. The most distinctive feature of insect haemolymph is, however, that it contains a much higher concentration of amino acids than does the vertebrate plasma. This high content of amino acids is best seen by the fact that free amino acids together contribute 40% of the total osmotic pressure of haemolymph, whereas in vertebrate plasma amino acids account for only 1% (Martignoni, 1960). Based on biochemical analyses of silkworm haemolymph, Wyatt (1956) designed a medium which was considered superior to any previously designed medium (Wyatt, 1956). Cells of the ovarian tissue of silkworm pupa grew and divided for weeks in this medium. Since then, a number of other insect tissue culture media were designed based on the haemolymph composition of the appropriate species (for examples: Schneider, 1964; Echalier and Ohanessian, 1970).
Even though the composition of haemolymph is a good guide for the design of insect tissue culture media, media so designed may not be appropriate for the nutritional requirements of some tissues from the insect. Insect nerve cells, for instance, are separated from the haemolymph by a blood-brain barrier. Media designed according to the contents of haemolymph sometimes fail to support very well the growth of insect neurons (Beadle and Hicks, 1985). In addition, most media designed have been based on the criterion of the ability of the medium to promote the rate of cell division, for the purpose of virus propagation or of establishing cell lines from culture (Grace, 1982), and this may not be suitable for the maintenance of primary neuronal cultures, or for the differentiation of cells from established cell lines. Another drawback of insect culture media is that most are not commercially available. Only two media can be purchased in their modified forms (Grace's medium for lepidopteran cells: Grace, 1962; and Schneider's *Drosophila* medium: Schneider, 1964).

Some investigators persisted in attempts to use vertebrate culture media for insect tissue culture, and in view of these concerns have successfully obtained insect tissue cultures with some vertebrate culture media. For instance, using trial and error, Chen and Levi-Montalcini (1969) devised a synthetic medium which supports vigorous neurite outgrowth from nervous tissues from cockroach embryos, by screening all commercially available media, including vertebrate media (Levi-Montalcini et al., 1973). This synthetic medium consists of five parts of Schneider's *Drosophila* medium and four parts of Eagle's basal medium ("4+5" medium), and gave better results than any other insect medium in supporting primary cultures of insect neurons (Levi-Montalcini et al., 1973; Beadle and Hicks, 1985; Lees and Beadle, 1988). The "4+5" medium has been used in embryonic and adult cell cultures and organ cultures of the cockroach nervous system (Levi-Montalcini, 1973; Beadle and Hicks, 1985). Another culture medium which has been widely used for insect tissue culture is a vertebrate medium, Leibovitz's L-15 medium (Wu et al., 1983). The ionic components of L-15 mixed with the non-ionic constituents of Grace's medium
(Stengl and Hildebrand, 1990) have been used for the culture of cells from the moth *Manduca sexta* (Hayashi and Hildebrand, 1990; Prugh et al., 1992).

**B. Drosophila culture media**

The widespread use of *Drosophila* in genetic approaches to physiology and development has meant that a variety of media have been designed and subsequently modified for culturing *Drosophila* cells and tissues. The development of media used in early studies has been summarized previously (Schneider and Blumenthal, 1978; Martin and Schneider, 1978), and the composition of five media widely used in early organ culture is provided by Martin and Schneider (1978). Subsequently, several new media have been devised (Shields and Sang; 1977, Davis and Shearn, 1976). The M3 medium devised by Shields and Sang (1977), with the supplementation of 10% fetal bovine serum, appears to have gained in popularity over other media (Dübendorfer, 1978; Lüer and Technau, 1992). This medium was further modified (MM3) by Edwards et al. (1978) so as to decrease the serum supplementation. The MM3 medium has, in particular, been claimed to give superior maintenance of imaginal discs and their cells (Milner and Haynie, 1979; Currie et al., 1988; Cullen and Milner, 1991). This medium, however, suffers the same disadvantage of not being commercially available. The vertebrate culture medium, Leibovitz's L-15 medium, which is commercially available, has also been successfully used to culture neurons in *Drosophila* (Wu et al., 1983).

### 1.4.3. Insect tissue culture

**A. Organ culture.** As in the history of vertebrate culture, the initiation of organ culture in invertebrates preceded the initiation of cell culture. Unlike vertebrate cultures, the small sizes of insects allowed most organs to be cultured entire. Organ cultures allow the direct observation of developmental events in an insect organ, which would not be possible with *in vivo* methods. Because intercellular or organotypic relations are retained among the
cells of the organ, it is easy to relate a particular group of cells with their *in vivo* counterparts. Unlike organotypic slice cultures of vertebrate nervous systems, however, organ cultures of insect nervous system do not have a uniform thickness, and as a result are usually more than several cell layers thick. It is not easy, therefore, to observe individual cells within the explanted nerve tissue. On the other hand, the imaginal discs of insects are more suitable, because they consist of cell sacs that are a simple epithelium. In organ cultures of imaginal discs, individual cells or cell clusters can be viewed without staining under the microscope using differential interference- or phase-contrast microscopy.

**B. Dissociated cell cultures.** Dissociated cell cultures grow in a monolayer, and, although the *in vivo* intercellular relationships are disrupted, such cultures enable observation of individual cells. Dissociated cell cultures, therefore, provide a level of microscopic resolution which is not attainable with organ cultures. Primary cultures from dissociated embryonic cells gives rise to divergent types of cell phenotypes, while those from dissociated postembryonic tissue usually contains more homogenous cell types. Cultures of heterogeneous cell populations can be used to study the interactions between cell populations. Using a modified Schneider's *Drosophila* medium, Seecof investigated the development and growth of primary cultures of dissociated embryonic cells from *Drosophila melanogaster*, and achieved the differentiation of both neuronal and muscle cells in culture (Seecof and Donady, 1972). Many studies of dissociated primary cultures have been used to study the events surrounding morphogenesis and metamorphosis (Dübendorfer and Eichenberger-Glinz, 1980), as well as the electrophysiological properties of neurons (Wu et al., 1983).

**C. Insect cell lines.** Tremendous efforts in cell culture have been devoted to the establishment and characterization of insect cell lines. Grace was the first to succeed in establishing insect cell lines from the ovaries of *Antheraea eucalypit* (Grace, 1962). Early
cell lines of *Drosophila* are listed in Schneider and Blumenthal (1978), and new lines have been established (Cullen and Milner, 1991). The characteristics of many cell lines are yet to be established (Sang, 1980).

**D. Culture techniques.** For both organ and cell cultures, similar culture techniques have been employed.

**a). The "hanging drop"/"hanging column" method.** This technique resembles the Maximow assembly used for organotypic culture of vertebrate tissues. In addition to Maximow depression slides, a number of other culture vessels have been designed to make "hanging drop" or "hanging column" cultures (Shields et al., 1975; Dübendorfer and Eichenberger-Glinz, 1980, Beadle and Lees, 1986). The technique permits the critical microscopic study of cultured cells and organs. The drawback to this technique is that it is complicated to change media, requiring the assembly to be disassembled.

**b). The "lying drop" method.** This method is a modification of conventional tissue culture methods. Instead of using the whole area of a culture vessel, the "lying drop" technique cultures cells or tissues in a small droplet of medium. Like conventional cultures, the cells and tissues in the culture can be observed with an inverted microscope. It is easy to change media using this method. The culture media are exposed to air, however, and evaporation changes the osmolarity in the small droplet of culture medium. This has to be overcome by using a humidified chamber in which to maintain cultures, or could also be overcome by adding more medium after the culture attaches to the substrate.

Both the "lying drop" and "hanging drop"/"hanging column" methods require only small amounts of culture medium. They are therefore suitable for supporting primary
cultures of insect cells which are not easily obtained in large quantities by dissection, as well as for culturing insect organs of small size.

c). Other techniques used. A number of culture methods using either "roller tubes" (Jones, 1962) or "culture bottles/flasks" (Waku et al., 1990) have been used for insect cell and tissue culture. The use of bottle and roller tubes permits the use of a large amount of medium, and therefore enables the culture of a large number of explants or cells. The "roller tube" technique also guarantees sufficient aeration of the culture medium, as well as cell interactions during the rotation. The roller tubes and culture bottles require large amounts of culture medium, though, so that these techniques are only appropriate for massive cultures.

To accomplish the objectives of the thesis stated above (p. 5) it is important to establish a primary culture system for the visual system of Drosophila, and study developmental events therein which are difficult to study in vivo. Occasional neurite outgrowth from eye discs has previously been reported by Milner (1980), but only in one case. This failure is possibly because of the poor attachment of eye discs to the culture substrate and because of the inconsistency of neurite outgrowth. Because eye-disc culture has not been explored further by other laboratories, a major goal of this thesis has been to establish a primary culture system for eye discs as well as for optic lobe cells. With this system, many aspects of visual system development can now be studied in a more controlled environment in vitro.
Chapter 2
MATERIALS AND METHODS

I. General Materials and Methods

2.1.1. Fly stocks and egg collection

Wild-type *Drosophila melanogaster* (Canton S) flies were used. Fly stocks were kept on blue medium (Carolina Biological) in milk bottles at 25°C with a 12 hour (h) light/dark cycle. The blue medium was supplemented with 5-10 grains of Fleischmann's active dry yeast per 10 ml to provide vitamins. Fungicide solution (See Appendices for composition) was added to the medium to prevent overgrowth of fungi in the blue medium.

Eggs were collected on fresh medium in large (100 x 15 mm) Petri dishes containing blue medium and yeast. Adult flies were removed from the Petri dish after 2-3 days after being anaesthetized by CO2. Second- or third-instar larvae or synchronized prepupae were selected for tissue culture according to the experimental design.

2.1.2. Preparation and timing of cultures

Timed pupae (white prepupae, 3h or 5h after pupariation), late third-instar or late second-instar larvae were used. Pupal stages are referred to as P+0 (white pupae), P+3 (3h after pupariation) or P+5 (5h after pupariation), respectively. It is necessary to express developmental stages in terms of hours after pupariation, rather than as percentages of the time until eclosion would occur, because the times for which cultures were held *in vitro* could not be related to the time for eclosion which did not occur. Because the period of white pupae (P+0) lasts only about one hour, the pupae timed in this way are well synchronized at this point. Although the white pupal colour may not indicate development with complete accuracy, it is nevertheless a decisive external feature and is reported as one of the markers for this stage of development (Ashburner, 1989, his Chapter 8). Third- and
second-instar larvae were selected according to the size of the larvae, as well as the size of eye-discs after dissection.

All procedures for culture preparation were performed under aseptic conditions. Pupae or larvae were cleansed of medium with distilled water and briefly surface sterilized with 70% alcohol, and were then transferred to a culture dish containing either culture medium or *Drosophila* Ringer's solution (see Appendices for composition). Dissection was carried out on a sterile (autoclaved) glass slide under a dissecting microscope. In a droplet of tissue culture medium, eye-antennal discs together with the cephalic complex were dissected out from animals by one of two ways: 1) Using one pair of forceps to hold the posterior end of the larva, the mouth parts were held and pulled out with another pair of forceps. As a result of this manoeuvre, the eye-antennal discs together with the cephalic complex were pulled out and separated from the rest of the larva. 2) The anterior-most tip of the mouthparts of the prepupa were cut open with a razor blade. Applying slight pressure on the pupal body with one pair of forceps, the eye-antennal discs with the cephalic complex were extruded from the cut anterior opening, and were separated from the rest of the body with a pair of dissecting needles fashioned from sharpened tungsten wire.

Eye-antennal discs attached to the cephalic complex were transferred to a second culture dish containing medium, for further dissection. Eye-discs or eye-antennal discs, and the lateral poles of the supraesophageal hemispheres (which contain the forming imaginal optic lobes) were separated from the cephalic complex with two tungsten needles to initiate cultures.

To reduce contamination from gut bacteria, the eye discs were washed several times with culture medium containing antibiotics (see 2.2.1).

In order to prevent the tissues from sticking to the glass, the pipette which was used to transfer eye-discs and optic lobes was soaked in fetal bovine serum (FBS) or culture medium containing 10% FBS for at least 2 minutes before using.
A. Eye-antennal disc culture. Eye-antennal discs were transferred individually with a fire-polished Pasteur pipette from the dissecting medium to the culture vessels, with a small droplet of about 10 µl culture medium, and were kept there for about one minute. The use of a small volume of culture medium increased the chance of attachment of the disc to the surface of the culture vessel. This step was executed with great care, because such small volumes of medium easily evaporated, especially in the laminar flow hood (Forma Scientific, Inc.). The culture medium was increased later, to a final volume of about 40 to 60 µl.

B. Eye-disc culture. Antennal discs were detached from eye discs using tungsten needles. The peripodial membrane was either retained or removed with sharpened tungsten needles. The resulting eye discs were transferred with a fire-polished pipette and cultured in the same way as for eye-antennal disc cultures.

C. Eye-disc fragment culture. Antennal discs and peripodial membranes were removed from eye discs as mentioned above. The resulting eye discs were further sectored into small fragments with tungsten needles. Fragments of discs were transferred into a sterile Eppendorf tube (1.5 ml) using a fire-polished pipette the tip of which was drawn out so as to give a small orifice, and washed thrice by sequential mixing with fresh medium, followed by centrifugation (4,000 rpm for 2 min) and decanting of the supernatant. After final centrifugation, fragments were suspended with 40-60 µl culture medium in the Eppendorf tube and plated into culture vessels (see below) with the fire-polished pipette. About 6 to 8 eye discs were used for each dish of fragment culture.

D. Dissociated cell cultures. Dissociated cell cultures were used to culture either eye-disc cells or optic lobe cells. Antennal discs and peripodial membranes were removed from eye discs. To facilitate enzymatic dissociation, two tungsten needles were used to cut the eye discs or lateral poles of the supraesophageal ganglia into as small pieces as possible (eye discs were cut into 20 pieces and the lateral poles of the ganglia into pieces
of the same size as eye fragments). The resulting tissue fragments were transferred into a sterile Eppendorf tube and centrifuged once. The supernatant was discarded, and the tissue fragments were re-suspended in Ca$^{2+}$-Mg$^{2+}$ free *Drosophila* Ringer's (Appendices). The supernatant was removed after a second centrifugation, and the tissue fragments were then suspended in 60 ml of either 0.25% trypsin (type XII-S, Sigma) or 0.05% collagenase (Gibco) in 1 mM ethylenediamine-tetraacetic acid (EDTA, Sigma) Ca$^{2+}$-Mg$^{2+}$ free *Drosophila* Ringer's, and were kept at room temperature for about 15 min. Enzymatic digestion was slowed by the addition of 60 ml of culture medium. Dissociation was facilitated by triturating the fragments through a fire-polished Pasteur pipette having an aperture size of 300 µm. Dissociated and partially dissociated cells were further washed three times with fresh culture medium, centrifuged and re-suspended, and were finally cultured with 40-60 µl culture medium in culture vessels.

**2.1.3. Culture vessels**

Two kinds of culture vessels were used: a) a square glass coverslip (8 x 8mm) resting in a small Petri dish (Nunc 1 53066, 35 x10 mm); b) a culture chamber, which was made of an aluminium slide with a hole of 8 mm in diameter in the middle, and with a coverslip glued on one side of the hole with Sylgard (Dow). After the culture was placed into the chamber, a second coverslip was used to seal the chamber from the open slide. The chamber therefore had the thickness of the aluminium slide, 1.5 mm.

Both the glass-coverslip and the culture chamber were precoated for at least 1h with 100 µg/ml poly-L-lysine (MW 30,000-70,000, Sigma), followed by a wash with sterilized distilled water, and were air-dried in the laminar flow hood prior to culture initiation.

Evaporation of the culture medium would have resulted in changes in its osmolarity. To prevent this happening, the coverslip carrying the cultured cells was surrounded by droplets of sterile *Drosophila* saline to humidify the culture environment. All preparations
were cultured in an upright droplet at about 23°C and in an ambient atmosphere, in a moisturized chamber, rather than a CO2 incubator.

2.1.4. Immunocytochemistry

Immunoreactivity to the following antibodies was examined: 1) 22C10, which recognises an uncharacterised epitope on membranes of *Drosophila* neurons (Fujita et al., 1982); 2) 24B10, which recognises the product of the gene *chaoptin* which encodes a member of the leucine-rich repeat family of proteins, and is a cell-specific adhesion molecule of *Drosophila* photoreceptors (Zipursky et al., 1985; Krantz and Zipursky, 1990); 3) anti-HRP, which is likely to recognise a 42K glycoprotein epitope on *Drosophila* neuronal membranes (Jan and Jan, 1982; Sun and Salvaterra, 1995); 4) anti-BrdU, which recognises the sites of incorporation of bromodeoxyuridine into replicating DNA (Gratzner, 1982) in dividing cells; 5) Rabbit anti-histamine polyclonal antibody, which recognises a histamine-like antigen (Pirvola et al., 1988).

A. DAB method. Cultures or cryostat sections of the head were fixed with the appropriate fixatives, the exact composition of which (see later) depend on the particular antigen to be detected. The specimens were rinsed with either 0.01M phosphate-buffered saline (PBS, see Appendices) or Tris-buffered saline (TBS, Appendices) twice (5 min each) and treated with 0.01% Triton-X 100 in buffer for 20 min. To block non-specific antibody binding, 3% normal goat serum (Gibco) was used. Primary antibody was added to the specimens. The specimens were left in primary antibody overnight at 4°C, or 3h at room temperature. After thoroughly washing the specimens with four changes of buffer for 20 min, secondary antibody conjugated with peroxidase was added to the specimens for 2h at room temperature. The specimen was then washed in a further four changes of buffer for 20 min. Next, buffer containing 0.3 mg/ml DAB (3,3′-diaminobenzidine, Sigma) and 0.01% **H**₂**O**₂ was added to the specimens. The reaction was monitored under the dissecting microscope. Cold buffer was used to stop the DAB reaction. After dehydration
with a graded ethanol series (30%, 50%, 70%, 90%, 95%, 100%, 100%, 5 min each), specimens were cleared in two changes of xylene (5 min each) and mounted in DPX mountant (BDH Chemicals).

**B. Fluorescence method.** To detect single antigens, the procedure was the same as for the DAB method, except that fluorescence-labeled secondary antibody was used instead of peroxidase-conjugated secondary antibody.

For double staining, two primary antibodies of different origin (rabbit or mouse antibody) were applied together to the specimens. After washing, corresponding secondary antibodies conjugated with either fluorescein isothiocyanate (FITC) or rhodamine (or indocarbocyanine, Cy3) were added together to the specimens.

Specimens were mounted in Vectashield™ mounting medium (Vector Laboratories, Inc.) after thorough washing, and were sealed with nail polish at the edges of the coverglass to prevent evaporation.

### 2.1.5. Light Microscopy

Living cultures in Petri dishes were examined with a Zeiss IM35 inverted microscope with a long working distance 16x dry, or 40x water-immersion lenses. Living cultures in their culture chamber and immunostained specimens were viewed in upright mode using a Zeiss Axiophot photomicroscope, from which images were ultimately photographed on 35mm film.

### 2.1.6. Morphometric measurements

To measure the length of neurites and the extent of pigmentation of cultured eye-discs, cultures were initiated in a culture chamber (see 2.1.3), and were then viewed in upright microscopic mode on a Zeiss Axiophot, using a 20/0.5 objective. Images were captured at daily intervals with a Hamamatsu CCD camera and Neotech image board into a Macintosh IIci computer. Using morphometric software (NIH Image 1.44): a) the density
of pigmentation was measured as the darkness of the image relative to the background density surrounding the culture fragment (which was set to 0); b) the lengths of neurites were measured with the aid of a keyboard mouse. For statistical comparisons between neurite lengths, a two-tailed unpaired t-test was performed using StatView 512+ (Brainpower, Inc.).

2.1.7. Scanning electron microscopy (SEM)

For SEM, cultures were established on glass coverslips in culture dishes (see 2.1.3).

Cultures were fixed with 2% glutaraldehyde in PBS for 30 min. After rinsing with PBS twice and 0.1 M sodium cacodylate (Sigma) once (2 min each), the cultures were post-fixed with 0.5% osmium tetroxide (EM Corp) in 0.1M sodium cacodylate for 15 min, followed by three washes in 0.1M sodium cacodylate, 5 min each. The culture preparations were then dehydrated in a graded ethanol series, as before but 15 min each.

The final change of 100% alcohol was replaced with a mixture of Peldri II (Ted Pella, Inc.) and absolute alcohol (in a ratio of 1:1) for 15 min, then 100% Peldri II for 15 min. The samples in Peldri II were then placed in a freezer (-20°C) to solidify the Peldri II quickly. Specimens in the solidified Peldri II were then put in a fume hood until the Peldri II completely sublimed and the specimens were critical-point dried. The dried preparations were then coated with platinum and examined with a Nanolab 2000 SEM (Bausch & Lomb).

The melting point of Peldri II is 24°C. The Peldri II had therefore to be liquified on a warm plate or in a water bath at 26-30°C before use, and all operation with Peldri II were performed at this temperature in the fume hood.

2.1.8. Transmission electron microscopy (TEM)

For TEM, cultures were directly established in plastic culture dishes (Nunc).
Cultures were fixed for 10 min in 2.5% paraformaldehyde in PBS and then 20 min in 2.5% glutaraldehyde in 0.1M cacodylate buffer. After rinsing with 0.1M cacodylate buffer, the cultures were post-fixed with 0.1% osmium tetroxide for 30 min. After a rinse in cacodylate buffer, the cultures were dehydrated in a graded ethanol series as before, 5 min each. The last change of 100% alcohol was replaced with a mixture of Polybed 810 epoxy (see Appendices) and absolute alcohol (in a ratio of 1:1 thoroughly agitated) for 30 min. The mixture was then replaced with pure Polybed 810 and kept at room temperature overnight. The culture preparations were changed to fresh epoxy the next morning and polymerized at 60°C for 48h.

Under a dissecting microscope, the area of interest was selected, and cut out. After mounting and trimming the block, ultrathin sections were cut with an Ultracut E (Reichert-Jung), and examined with a Philips 201C TEM after post-staining in saturated aqueous uranyl acetate and lead citrate (see Appendices) for 10 min each.
II. Culture conditions

Wild-type Drosophila melanogaster (Canton S) flies were used in this study. Culture media were examined for their ability to support healthy cultures both of eye-imaginal discs from either late-third instar larvae or prepupae, and of optic lobe cells.

2.2.1. Culture media

The following culture media were tested:

1) Schneider's Drosophila medium (Gibco, 11720-018) supplemented with 10% or 20% heat-inactivated fetal bovine serum (FBS, Gibco, cat. no. 16000-010), 10 mM Bis-Tris buffer, pH 6.8. Heat-inactivation was achieved by treating the serum in a 56°C water bath for 30 minutes.

2) "4+5" medium, prepared by mixing four parts of Schneider's Drosophila medium with five parts of basal Eagle's medium (Gibco), supplemented with 10 or 20% heat-inactivated FBS (following the practice of previous investigators: Levi-Montalcini, 1973; Beadle and Hicks, 1985), 10 mM Bis-Tris buffer, pH 6.8.

3) Leibovitz's L-15 medium (Gibco) supplemented with 10% heat-inactivated FBS (following the practice of previous investigators: Wu et al., 1983), 10 mM Bis-Tris buffer, pH 6.8.

4) A mixture of 27% Leibovitz's L-15 medium and 73% Drosophila Ringer's solution, but with the omission of bicarbonate (see Appendices for composition, modified from Wu et al., 1983) supplemented with either 5% or 10% heat-inactivated FBS, 10 mM Bis-Tris buffer, pH 6.8. (10% FBS was used to conform with other media used by previous workers, and 5% was used in an attempt to use this medium as closely as possible as a defined medium.)

5) Shields and Sang's M3 modified medium (MM3) (see Appendices, from Cullen and Milner, 1991) with the addition of 2% FBS (following the practice of Cullen and
Milner, 1991), 10 mM Bis-Tris buffer, pH 6.8. This medium was prepared in the lab, with all ingredients from SIGMA except L-glutamine (Gibco) and T.C. Yeastolate (Difco). The detailed composition of the medium is listed in the Appendices.

All media listed above contain 50 mg/L penicillin G (Gibco) and 100 mg/L streptomycine (Gibco) to inhibit bacterial growth. To test the effects of including steroid moulting hormone, 20-hydroxyecdysone (20-HE: Sigma, cat. No. H-5142) was added to the medium as noted, to a final concentration of 1 µg/ml.

The pH was 6.8 for all media tested, matching the pH of fly haemolymph. Different buffer systems were used in each medium. The original buffer for Schneider's and Culture medium was changed every three or four days by adding 60 µl of new medium and withdrawing an equivalent volume of the mixture.

2.2.2. Initial evaluation of culture media

In order to permit successful cultures to be made, a medium had to support the attachment and survival of cultures for a prolonged period. Culture media were evaluated by the period of time it took for 50% of the eye disc fragments to detach from the culture substrate (the T1/2). Poly-L-lysine coated coverglasses were used as the substrate. Cultures were examined with a Zeiss IM35 inverted microscope. The attachment of disc fragments to the substrate was examined by lightly tapping the microscope stage with the finger tip. Fragments attached to the substrate did not move under this vibration, while fragments that had become detached did move, even if their neurites may still have been attached to the substrate. The total number of disc fragments when the cultures were initiated, the number of detached fragments, and the number of adhering fragments, were all recorded daily. Selected cultures were photographed with differential interference contrast optics at an objective magnification of 16X.
2.2.3. Morphological changes and pigmentation

To examine the ability of the culture medium to support cellular differentiation of eye-imaginal discs in vitro, neurite outgrowth, the development of screening pigments, and overall morphological changes of both entire discs and eye-disc fragments in culture, were all observed and photographed on 35 mm negatives as mentioned in section 2.1.5. Neurite length and pigmentation were measured as in section 2.1.6.

2.2.4. BrdU incorporation

Mitotic activity of cultured cells and their counterparts in vivo were examined by BrdU incorporation.

A. The incorporation of BrdU in vivo. The in vivo incorporation of BrdU was obtained after applying BrdU (5-bromo-2'-deoxyuridine, Sigma) to the larval body wall for 15 min in a 1:1 mixture of DMSO (dimethyl sulfoxide, Sigma) and acetone (Winberg et al., 1992). BrdU was applied at a concentration of 0.5% to white prepupae while the cuticle was still soft. These prepupae were then kept on fly medium for five hours, at which point the cephalic complex including the eye discs were dissected out and fixed with 2% paraformaldehyde in PBS.

B. The incorporation of BrdU in vitro. For BrdU incorporation in vitro, cultures were initiated on coverslips in a 35 mm Petri dish (See 2.1.3). To examine the mitotic activities of cultures after different culture durations, tissue culture medium containing 1μM BrdU was either used to initiate the culture, or either eye discs or optic lobe cells were first cultured in a medium with no BrdU and the medium was then replaced with one containing 1μM BrdU for different periods. Cultures were then washed with PBS and fixed with 2% paraformaldehyde for 20 min.

BrdU incorporations were visualized immunocytochemically (Gratzner, 1982). Fixed cultures and eye discs from intact animals were washed with PBS, treated with 1.0 N HCl for 45 min to denature nuclear DNA, followed by neutralization with 1mM sodium
borate (Cattaneo and McKay, 1990). A DAB method (2.1.4A) was used for labeling BrdU positive cells. The primary and secondary antibodies used were mouse monoclonal anti-BrdU antibody (Becton Dickinson), diluted 1:50, and peroxidase-conjugated goat anti-mouse secondary antibody (Bio-Rad Laboratories), also 1:50. The buffer used was PBS (pH 7.0). Non-dividing cells in the culture were used as negative control. In some cases, the cultures were counterstained with 0.1% neutral red to visualize BrdU-negative cells.

2.2.5. Movement of the morphogenetic furrow

Movement of the furrow was examined by continued direct observation, and as well was indirectly inferred from double labeling methods, as follows.

A. Direct observation. Movement of the morphogenetic furrow was detected relative to a hole that had been made in a cultured eye disc with the tip of a micropipette previously coated with crystals from an evaporated saturated ethanolic solution of the lipophilic dye, diI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, DiIC<sub>18</sub>(3), Molecular Probes, cat. no. D282). The dye was used only to localize a reference point, relative to which the position of the furrow could be detected as different times to reveal its possible movement.

B. Double labeling. Early third-instar larvae were used for a double-labelling experiment. The in vivo BrdU labeling method (See section 2.2.4A) was used to detect the second mitotic wave of the eye discs, except that the concentration of BrdU was increased to 50 mg/ml and the larvae were kept on fly food for two hours only, before dissecting eye discs for culture. Whole eye discs with the peripodial membrane attached were then removed and cultured for 10h. At this point, eye discs were fixed for BrdU immunocytochemistry as described in section 2.2.4, except that FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories) 1:50 was used instead of the corresponding peroxidase-conjugated antibodies.
Phalloidin stains actin filaments and is useful for recognizing ommatidial preciusters (Wolff and Ready, 1991). For double-stained preparations, at the conclusion of BrdU staining after incubation in FITC-conjugated goat anti-mouse antibody, the eye discs were thoroughly washed with PBS, and then put in 0.1 mM tetramethylrhodamino-isothiocyanate (TRITC)-phalloidin (Sigma) in PBS and kept overnight at 4°C. Phalloidin and BrdU stained patterns were finally examined using a Zeiss LSM410 confocal microscope.

2.2.6. Cell viability detection

To detect living cells in culture 1 mM calcein AM (Molecular Probes) was used. This penetrates membranes and is hydrolyzed by intracellular esterases in living cells. To detect the nuclei of dead cells, 1 mg/ml ethidium bromide (Molecular Probes) was used, which penetrates only those cells with membrane permeability defects, and binds to DNA. Cells were examined within 30 min of exposure to a mixture of the two dyes, and photographed with epi-fluorescence on a Zeiss Axiophot.

2.2.7. Nuclear staining

Living cultures were washed with PBS, stained with 0.5 mg/ml Hoechst 33258 (Molecular Probes), and examined by UV fluorescence. The diameters of stained nuclei and of spherules (see Results) were measured by image analysis as in section 2.1.6.

III. Effects of 20-hydroxyecdysone on eye-disc differentiation

2.3.1. Fly stocks

In this study, wild-type Drosophila melanogaster (Canton S) flies were used in all the experiments except those on pigmentation. To study the formation in vitro of the screening pigments, ommochromes and drosopterines, eye-colour mutants, brown (bw)
and vermilion (v) were used, obtained from the National *Drosophila* Species Resource Center at Bowling Green State University. Synchronized white prepupae were selected for tissue culture at either 3h (P+3) or 5h (P+5) after pupariation.

### 2.3.2. Establishment of eye-disc cultures

Eye-disc fragment cultures were established as in section 2.1.2C. The effects of 20-HE were studied by comparing results from cultures supplemented with 1 μg/ml 20-HE with those from cultures lacking 20-HE.

### 2.3.3. Immunocytochemistry

**A. Neuronal antigens.** Neuronal differentiation was examined by detecting the presence of antigens for anti-HRP (Jan and Jan, 1982, Jackson ImmunoResearch Laboratories, Inc.) as well as for monoclonal antibody 22C10 (Fujita et al., 1982; Zipursky et al., 1984; gift of Dr. S. Benzer, CalTech). Rabbit anti-peroxidase (1:1000) (Jackson ImmunoResearch Laboratories, Inc.) was used for anti-HRP immunoreaction, using the DAB method described in section 2.1.4A. The buffer used was PBS. For 22C10 immunostaining, primary antibody was used at a dilution of 1:1, and TBS was used as a buffer. The immuno-fluorescent method given in section 2.1.4B was used to visualize the immunoreactivity.

**B. Photoreceptor-specific antigen.** Differentiation of photoreceptors was indicated by the expression of antigen for monoclonal antibody 24B10 (Fujita et al., 1982; Zipursky et al., 1984; gift of Dr. S. Benzer, CalTech). The procedure for immuno-staining the antigen was the same as for 22C10.

**C. Histamine immunoreactivity.** To examine the expression of the normal neurotransmitter for the photoreceptor cells, histamine (Hardie, 1987, 1988), special fixation was used for immunoreactivity to anti-histamine.
a). Cryosection of pharate-adult and pupal heads. Heads of pharate adults (those about to eclose) and of pupae at the stage of eye development described as peach-eyed (about 50% pupal development, Cagan and Ready, 1989) were dissected in 4% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCDI, Sigma E 7750) in PBS (Pirvola, et al., 1988). After removing the mouth parts, the heads were fixed in 4% EDCDI for 2.5h before being transferred to 20% sucrose in PBS overnight at 4°C. Horizontal sections were cut at 14 μm with a 2800 Frigocut N (Reichert-Jung), and were collected on slides pre-coated with gelatin.

b). Fixation of cultures. Eye-disc fragment cultures (SIV) were fixed with 4% EDCDI for 10 min, followed by fixation in 2% paraformaldehyde in PBS for a further 10 min.

An immuno-fluorescent method (section 2.1.4B) was used for both head sections and cultured eye-disc fragments. The primary antibody, rabbit-anti-histamine (1:1000) was a gift from Dr. P. Panula (Helsinki).

D. Synaptotagmin expression. Rabbit anti-synaptotagmin (DSYT2) (gift of Drs. T. Littleton and H. Bellen, University of Texas) was used at 1:1000 dilution to detect the expression of a synaptic vesicle protein, synaptotagmin. Cultured eye-disc fragments were fixed and double stained with DSYT2 and 224B10, using procedures described in section 2.1.4B.

2.3.4. Morphometric measurements and dose-response curves

Neurite lengths of cultured eye-disc fragments in different 20-HE concentrations were measured and statistically compared, as in section 2.1.6. Dose-response curves for neurite lengths were constructed in two series of concentrations of 20-HE at 0, 0.5, 1.0, and 2.0 mg/ml and of 0, 0.125, 0.25, and 0.5 mg/ml. These were plotted using DeltaGraph™ v1.5 software.
2.3.5. Preparation of fly extract

Fly extract was prepared according to a protocol obtained from Dr. P. Milner (personal communication) with some modification. Newly eclosed flies were anaesthetized on ice. About 100 flies were added to a 1.5 ml Ependorff tube with 1 ml of MM3 medium without the supplementation of serum, and homogenized on ice with a homogenizer (Glas-Col). The homogenate was transferred to a 10 ml centrifuge tube and centrifuged at 150 g at 4°C for 30 min. The supernatant was transferred to a new tube. After removing the oily layer at the top of the supernatant, centrifugation was carried out as above for 1.5 hour. The resulting supernatant was sterilized with a 0.2μm Millipore filter and stored at -20°C.

2.3.6. Non-neuronal differentiation

Formation of pigment, lens and interommatidial bristles was examined directly by light microscopy (section 2.1.5). The spreading of glia-like outgrowths from cultures was examined by scanning electron microscopy (SEM) (section 2.1.7).

IV. Regenerating photoreceptor axons in vitro and their interaction with optic lobe cells

2.4.1. Observation of regenerating photoreceptor axons

Eye discs from P+5 were dissected as in section 2.1.2C. The fragments were then washed with Ca²⁺-Mg²⁺ free Drosophila Ringer's, and treated with 0.05% trypsin for 10-15 min at room temperature. After trituration and washing, with centrifugation, the partially dissociated disc fragments were cultured in culture chambers with poly-L-lysine as a substrate. Immediately after culture initiation, exposed axonal stumps were examined with a Zeiss IM35 inverted microscope and photographed with a 35 mm camera. Attachment and elongation of the severed axons was recorded at different time intervals.
2.4.2. Influence of histamine on neurite extension

The lengths of neurites in cultures were measured in medium containing a dilute solution of histamine, as in section 2.1.6. The concentrations of histamine used in this experiment were 0, 0.1, 0.5, or 1.0mM. Eye-disc fragments from P+5 were cultured in medium from this concentration series for 4 days and 6 days.

2.4.3. Examination of synaptic structure

Co-cultures were initiated by culturing eye-disc fragments (section 2.1.2.C) and dissociated optic lobe cells (section 2.1.2.D) together. Synaptic structures were examined with transmission electron microscopy (TEM) as described in section 2.1.8.
Chapter 3
RESULTS

I. Culture conditions

The first prerequisite for obtaining successful cultures was to identify the appropriate culture conditions. These included the culture medium to be used, the proper temperature of the culture environment, and the appropriate pH, required to support the survival and growth of cultures. Previous work on insect tissue culture (see Chapter 1) has seemed to agree on two parameters: the temperature for maintaining the cultures, which was chosen as room temperature (i.e. the temperature at which the insects were reared); and the pH, which was usually selected according to the physiological value of the haemolymph (Echalier and Ohanessian, 1970). The selection of the culture medium, however, has varied, as mentioned in Chapter 1. Therefore, testing a variety of culture media was necessary, in order to find the one which could support the survival and neurite outgrowth from eye-imaginal discs as well as from the optic lobe cells. To establish a primary culture system thus became the first and most important step of the entire project, and this, it is hoped, will be a lasting contribution of this work. The studies presented in this section have focused on selection of the culture medium and characterization of the survival and differentiation of cultures in the selected culture medium.

In order to perform a manageable number of trials, the selection of media was limited to those used previously in postembryonic neuronal cultures. Five combinations of three culture media that had previously been successfully used for culturing postembryonic neurons and organs were tested: Schneider's Drosophila medium, previously used to study the differentiation of eye-antennal discs which were still attached to the cephalic complex (Schneider, 1963, 1964); the "4+5" medium, which has been used to culture the adult central nervous system of the cockroach (Levi-Montalcini et al., 1973; Howes and Leech, 1985); Leibovitz's L-15 medium, used to culture Drosophila postembryonic...
neurons (Wu et al., 1983); and the medium MM3 which has been used for studies of 
imaginal disc differentiation (Currie et al., 1988; Cullen and Milner, 1991). Initial trials 
indicated that MM3 medium with the supplementation of 2% FBS was the best medium 
tested. This medium has then been the subject of further evaluation.

In initial cultures established in both Schneider's and in "4+5" media the addition of a 
supplement of insulin (100ng/ml) did not appear to influence the survival of culture 
fragments. These culture conditions were however poor, so that no conclusion can be 
reached about the possibly beneficial effects of insulin. In later cultures using the MM3 
medium, the survival of cultures was sufficiently improved without insulin, that an insulin 
supplement was not tested further (see Conclusions, below).

3.1.1. Culture Media

Five combinations of three media (section 2.2.1) were tested for their ability to 
promote good survival and neurite outgrowth from eye-imaginal discs as well as from optic 
lobe cells. The cultures were maintained at 23-25°C, the temperature range at which fly 
stocks were maintained in the laboratory. The pH was set at 6.8 according to the 
physiological value obtained for Drosophila haemolymph (Begg and Cruickshank, 1963).

A. Schneider's Drosophila medium and "4+5" medium

Eye discs and optic lobe cells were not healthy in either the four cultures established 
in Schneider's medium or the six cultures in the "4+5" medium, each with about 10 
fragments per culture, i.e. matter if they were cultured with, or without, supplementation 
with 10-20% heat-inactivated FBS. The discs began to darken during culture, by 
comparison with their translucent appearance when taken fresh from larvae. They also 
detached from the substrate quickly, and neurite outgrowth did not commence. Shortly 
after initiating the cultures, precipitates started to form on the bottom of the culture dish. 
These could be dissolved by the addition of HCl and reformed by adding NaOH,
suggesting that the formation of precipitates was possibly due to a pH change. This was perhaps caused by the loss of CO\textsubscript{2} from the culture media, since the commercial forms of Schneider's and basal Eagle's media both contain bicarbonate buffers designed for use with a CO\textsubscript{2} incubator. Although 10 mM Bis-Tris was added as a buffer in the "4+5" medium, this supplement was apparently insufficient to offset the pH change caused by CO\textsubscript{2} loss from the medium. Therefore Schneider's Drosophila medium and the "4+5" medium were not used for further studies.

B. Leibovitz's L-15 medium, and its mixture with 73% Drosophila Ringer's solution

These two combinations of media when supplemented with 10% FBS supported the survival and differentiation of neurons from cultures of eye-discs and optic lobe cells. Neurite extension from both cultures was observed within a few hours of initiating the cultures. In general, the optic lobe cells seemed less demanding than eye-imaginal discs under all culture conditions. They survived and differentiated very well in these two combinations of media as well as in the MM3 medium, which will be discussed below. Cultures of optic lobe cells could be maintained in these two media up to 30 days before most died.

The eye-discs were more exacting in their requirements of culture conditions. The survival of eye-disc cultures in these two combinations of media was inferior to that of optic lobe cell cultures, even though neurite outgrowth from eye discs was observed in both media. The T\textsubscript{1/2}, the time taken for 50% of eye-disc fragments to detach from the substrate, estimated from three cultures (10-15 fragments per culture), was 5-6 days for L-15 when supplemented with 10% heat-inactivated FBS (Fig. 1). About 35% of eye-disc fragments were still attached to the substrate after 9 days in culture. The attachment became weak, however, and varicosities had appeared along the extended neurites after these prolonged culture periods, suggesting the poor health of the cells.
Eye-disc fragments also looked healthy and gave abundant neurite outgrowth in a mixture of 27% L-15 and 73% *Drosophila* Ringer's solution supplemented with 5% FBS, but cultures in this medium were not studied systematically.

C. *Shields and Sang's M3 modified medium* (MM3)

MM3 medium with 2% FBS gave the best survival of eye discs and disc fragments. The $T_{1/2}$ which was calculated from five cultures (about 10-15 fragments per culture) was 11-12 days (Fig. 1). About 30% of eye-disc fragments remained attached to the substrate 14 days after initiating cultures, and some cultures were maintained for 20 days or longer. The difference of $T_{1/2}$ between the MM3 and L-15 media has not been examined statistically, because the small sample number. The survival and differentiation of optic lobe cells in 2% FBS MM3 was comparable to that in 10% FBS L-15 or its mixture with saline. The MM3 medium was therefore chosen for further studies.
Fig. 1. Attachment of P+5 eye-disc fragments to the substrate during varying periods of culture. Filled circles: MM3 supplemented with 2% FBS; open circles: L-15 medium supplemented with 10% FBS.
The longer T1/2 times in the MM3 medium, with 2% FBS supplement, compared with those in L-15, with 10% FBS, might have been attributed to differences in the FBS content, rather than to other components of the two media. For example, FBS might compete for the cells' binding sites to the substrate. This is not the case, however, because after initiating cultures in both media, all fragments attached to the substrate within 10h (for sample numbers, see above). Thus the cells had similar adhesivity in the two media, and the shorter T1/2 times in L-15 (Fig. 1) are attributable to poorer viability of fragments in this medium.

Cultures of eye-disc fragments (see section 2.1.2C) grown either in MM3 or in L-15 media developed a cuticle-like covering that formed over the eye-disc fragment, as previously observed by Gottschewski (1960) in cultures using a medium of his own devising. The difference between the two media was that the cuticle-like covering formed in L-15 had one or more large vesicles that bulged outwards from the fragment. By contrast, the cuticle-like secretion that formed over cultures in the MM3 medium with 2% FBS usually covered the eye-disc fragment without forming vesicular blisters.

3.1.2. Cultures of eye discs

A Types of culture

Three types of cultures were established for eye-discs: entire eye-antennal or eye disc cultures, eye-disc fragment cultures, and dissociated eye-disc cultures. The former two were designed to obtain neurite outgrowth from the photoreceptors of an eye disc in a situation that preserved as closely as possible the original geometry of cells in the disc. Eye-disc cultures were established in 2% FBS MM3 medium.

Eye-antennal and eye disc cultures. In order to achieve neurite outgrowth from imaginal discs it was first necessary to obtain preparations that attached to the bottom of the culture vessels. This was relatively difficult with eye-antennal discs and eye discs,
which tended to float in the medium. Several reasons could have underlain this problem. Possibly the outer surface of the cells lacked sufficient adhesivity, but the convex shape of both the eye-antennal and eye discs also allowed only a small area of contact with the substrate. Using the culture techniques for eye-antenna and eye disc cultures described in sections 2.1.2A and 2.1.2B, the chances of the disc touching the culture substrate were greatly increased, and the probability for disc attachment although still not great was increased to at least 20% (in at least four cultures each of at least five discs, the worst case was for only disc to adhere to the substrate). Eye-antennal discs did not adhere as well as eye discs from which the antennal disc had been severed, possibly because they were more convex. Neurite outgrowth was observed from both the eye-antennal and eye discs, but it was not profuse.

The adhesion of the eye disc to the culture substrate was greatly increased by removing the peripodial membrane. After 3 days in vitro (3DIV), the representative disc in Fig. 2A had obvious neurite outgrowth from the posterior end of the disc at two sites, the optic stalk and a site of possible rupture. Both sites had an epithelium-like outgrowth from which neurites extended. Because the culture medium contained 1µg/ml 20-HE (see 3.2.4, below), pigmentation had initiated at the edge of the disc (Fig. 2A). The shape of a second eye disc still with its peripodial membrane attached (Fig. 2B) was more convex than the first. The folds in the disc typical of this type of culture indicate that it only partially adhered to the substrate. Neurite extension from such discs was moreover not consistent, and was usually seen only in discs which had an expanded surface at the tip of the optic stalk (Fig. 2B). The latter probably arose from the stalk epithelium, which therefore may have provided an intermediate substrate from which neurites could gain access to the surface of the culture dish. In these two cases (with and without the peripodial membrane), the number and length of neurites were both inferior to those from eye-disc fragments (see below).
Fig. 2. Neurite outgrowth from entire discs. (A) A disc from P+5 with peripodial membrane removed, 3DIV. Neurite outgrowth is restricted to the posterior margin, at the optic stalk (os) and nearby, at a site of possible rupture in the disc (arrow head). Pigmentation has initiated at the edge of the disc. (B) A disc from third instar with peripodial membrane attached, 1DIV. Neurite outgrowth is seen at the extended optic stalk. Live culture, differential interference contrast. Anterior lies to the right of the figure. Scale bar: 50μm.
Eye-disc cultures have been followed for at least two weeks. Despite the length of time these cultures could be maintained, and notwithstanding the fact that cultures of whole discs allowed photoreceptor cells to develop under the influence of their normal cell neighbours, much as they would do in vivo, the cultures were unsatisfactory: restricted and inconsistent extent of neurite outgrowth remained a major limitation for studies concerning this aspect of photoreceptor differentiation. This limitation persisted under all the many culture conditions that were tried, so that further attempts concentrated upon eye-disc fragments and on dissociated cells, to try to achieve more consistent neurite outgrowth.

**Dissociated eye-disc cultures.** In cultures of dissociated cells from eye imaginal discs, most cultures existed as small cell clusters of various size. In marked contrast to the general lack of outgrowth observed from the entire discs, most cell clusters developed neurites. Such cultures are useful for studies on cell growth, on neurite outgrowth and on the fasciculation of neurites, and all occur in isolation from their normal eye disc cells in vivo. In typical examples after being cultured for five days, the neurites extended and fasciculated to form fibres more than 100 μm long (Fig. 3). From their expression of a neuronal marker recognized by antibody directed against HRP (Jan and Jan, 1982), it could be confirmed that these cells were in fact neuronal (Fig. 3). Only the limited relationships between neighbouring cells within the same cell cluster could have been preserved, however. The overall spatial relationships that existed among the cells in the disc in vivo no longer existed in such dissociated cell cultures. Moreover, the survival of dissociated eye-disc cells was poor compared with disc fragment cultures (see below). The number of surviving cells immediately after cultures were initiated was fewer, and neurite outgrowth was less frequent, although cells that survived were able to extend long neurites. Because of the traumatizing effects of enzymatic dissociation and mechanical trituration, an appreciable amount of cell debris existed in the dissociated cultures. Large clusters (Fig. 3A,B) resulting from partial dissociation survived better, however, with
Fig. 3. Dissociated photoreceptor cells from third instar larvae cultured 5 DIV in L-15 supplemented with 10% FBS. (C, D) Cells in the small clusters emit neurites that fasciculate forming a long fibre. (A, B) Larger clusters have neurites extending from several locations. Stained with anti-HRP. Scale bar: 20μm.
healthy neurite outgrowth. For this reason, a compromise culture condition utilizing eye-disc fragments was adopted.

**Cultures of eye-disc fragments.** Cultures of fragmented eye discs not only retained most of the local cellular spatial relationships of the disc *in vivo*, they also gave rise to vigorous neurite outgrowth (Fig. 4). This outgrowth has not previously been reported. Within several hours after the cultures were initiated, nearly all eye-disc fragments were adhering to the substrate. This culture system allowed repeatable observations on vigorous neurite outgrowth and other aspects of cellular differentiation, as well as on regeneration, so that most of the studies reported here utilized this type of culture.

Fragments of eye discs exhibited different shapes in culture depending on the age of the animal at the time of culture preparation. Disc fragments from animals younger than 3h post-pupariation (P+3) usually sealed into a round vesicular form (Fig. 4), while fragments from discs of animals at P+5 or older often maintained the shape of the fragment after dissection (Fig. 5). In both stages, the morphology of the disc fragment changed as the culture continued, implying the possibility of morphogenesis *in vitro*. Freshly dissected eye discs had ommatidial patterns visible under differential interference contrast optics. These ommatidial patterns were retained during development *in vitro* in some disc fragments from P+5 (Fig. 5). On the other hand, no ommatidial patterning was observed in cultures from pupae at P+3 or younger. Because eye-imaginal discs from late third-instar larvae already exhibit clear ommatidial patterning (Ready et al., 1976), these patterns must also have been present in eye-discs from P+3 pupae. The rounded shapes adopted by the disc fragments from these younger animals, however, made the resolution of any such patterns too difficult by differential interference contrast optics.

Neurite outgrowth from disc fragments started within five hours of initiating cultures from P+3 and P+5 pupae. Lamelliform structures that clearly differed from the fibre outgrowth of neurons, and which may therefore have originated from a non-neuronal
Fig. 4. P+3 eye-disc fragment cultured after 3 DIV in MM3 with 2% FBS. The fragment has formed into a vesicular shape. Abundant neurites emerge from the fragment, fasciculating and defasciculating extensively to give the branched appearance. Live culture, photographed under differential interference contrast but through the plastic substrate, not under optimal optical conditions. Scale bar: 30μm.
Fig. 5. P+5 eye-disc fragment after 3 DIV in MM3 with 2% FBS. Ommatidial array is clearly preserved, and shape of fragment after dissection retained. Neurite outgrowth is obvious, but in a different focal plane. Live culture, photographed under differential interference contrast but through the plastic substrate, not under optimal optical conditions. Scale bar: 20μm.
eye-disc epithelial cell, were also often seen extending from disc fragments. From the sites of such sheets of presumed non-neuronal origin, neurites invariably extended (Fig. 6A). In some cases, the sheets appeared very soon after the initiation of the culture, before neurite outgrowth, indicating that the sheets were possibly the substrate for the neurites and not vice versa. Frequently, neurites also grew at sites elsewhere in the culture, or from cultures lacking such sheets (Fig. 6B). Immunoreactivity to the monoclonal antibody 24B10 (Fujita et al., 1982) indicated that these neurites were photoreceptor axons (Fig. 7). Optic lobe cells failed to exhibit such immunoreactivity, and thus serve as a control for the photoreceptor-specific staining of these cultures. At their tips, neurites had clear growth cones with filopodia and, along their length, occasional swellings. The latter were not clear in living neurites, viewed at lower magnification, however (Figs. 4, 6), and could therefore have been caused by osmotic shock during the immuno-staining procedure, presumably during fixation with 2% paraformaldehyde in 0.01M PBS.

The age at which cultures were initiated did not influence neurite outgrowth from eye-disc fragments, at least at early pupal stages. Vigorous outgrowth was seen in cultures from both P+3 and P+5 pupae. The average neurite lengths for P+3 cultures after 2DIV and 4DIV were similar to those of P+5 cultures. The average length at 2DIV was about 120 μm and at 5DIV was about 130 μm for both P+3 and P+5 (Fig. 8).

B. Requirement for 20-HE in culture conditions

The central role played by 20-HE in the differentiation of insect cells in vivo (Truman et al., 1993) and in vitro (e.g. Mandaron, 1971; Fristrom et al., 1973; Milner and Sang, 1974; Prugh et al., 1992), suggested the importance of testing the effect of this hormone on the pigmentation and other aspects of development of eye discs in culture (see Results II). Pigmentation can be scored easily, and was therefore used as a criterion to examine the necessity of including 20-HE in the culture medium. In addition, it could be
Fig. 6. P+3 eye-disc fragments 1DIV in MM3 with 2% FBS. (A) Extending from the edge of the explant are wide sheet-like expansions shown in the left of the figure. Outgrowth of the more slender neurites occurs from these non-neuronal sheets. (B) Neurite outgrowth from a culture lacking these non-neuronal sheets. Live cultures, differential interference contrast. Scale bar: 30μm.
Fig. 7. Neurite outgrowth from eye-disc fragment arises from photoreceptors. Cell bodies within the fragment and extending neurites are 24B10-immunoreactive. Varicosities occur along the extending neurites (arrowhead) and growth cones arise at the neurite tips (arrow). P+5 eye-disc fragment 3DIV in MM3 with 2% FBS. Scale bar: 20µm (inset: 100µm).
Fig. 8. Average neurite lengths (± SD) from P+3 and P+5 eye-disc fragments at 2DIV and 4DIV. There is no significant difference between P+3 and P+5. Numbers above bars are numbers of culture fragments.
used to examine how important was the effects of the donor animal's age in obtaining close to normal development of tissues *in vitro*.

Pigmentation was seen in all intact disc and disc fragment cultures that were supplemented with 20-HE. When cultured in a medium containing 1 μg/ml 20-HE, for example, eye-disc fragments from P+5 pupae, which were initially unpigmented, started to exhibit pale yellow pigmentation after two days in culture. This pigmentation darkened with duration of subsequent culture (Fig. 9). The first appearance of this screening pigment (Fig. 10) at about 2 days *in vitro* (DIV) is similar to the earliest time at which ommochrome pigmentation is detected *in vivo* (Shoup, 1966). Thus, culturing eye-disc fragments from P+5 pupae did not delay the onset of pigmentation. Pigment was not evenly distributed in the cultured discs, but exhibited patches of darker pigmentation surrounded by paler areas (Fig. 10). These levels of pigmentation were not compared photometrically with the corresponding levels *in vivo*. No detectable pigmentation was seen in discs cultured up to 10DIV in a medium lacking 20-HE. The darkest region of the patches of darker pigmentation was set to 100% pigmentation, so that maximum pigmentation averaged about 70% (Fig. 9).

The commencement of pigmentation *in vitro* was affected by the age of the animal at the time at which cultures were prepared. When eye discs were taken from P+3 pupae, only two hours younger than P+5, pigmentation was delayed for about 10h. Late third-instar larvae showed pigmentation only after 72-96 h and discs from early third-instar larvae took up to 240h in culture to start pigmentation. By contrast, eye-disc fragments from older animals exhibited pigmentation after only short culture durations. (See section 4.1.3 for discussion). For example P+24 pupae displayed pigmentation after about 24h in culture, at an aggregate time (48h post pupariation) similar to the first appearance of pigment *in vivo* (Shoup, 1966). Neurite extension was infrequent and neurite length was short from eye-disc fragments from P+24 pupae (Fig. 11).
Fig. 9. Pigmentation from P+5 eye-disc fragments darkens as the culture continue. Four cultured fragments were each followed for 5 days.

Pigmentation (pho\textsuperscript{m}etrically measured from image analysis) was measured in two locations on each fragment -- the more deeply pigmented patches ('patch') and the paler surrounding regions of the fragment ('whole fragment': see Fig. 10). Both darken as culture life continues.
Fig. 10. Pigmentation of eye-disc fragment culture in the presence of 20-HE (1μg/ml). P+5 fragment cultured for 78h in vitro. Pigmentation is stronger at one point than in the surrounding disc. Live culture, differential interference contrast. Scale bar: 15μm.

Fig. 14. Advance of the area of pattern formation during a 10h period in vitro in confocal image of an early third-instar disc. A band of nuclei (FITC-BrdU: green) that had incorporated BrdU in vivo corresponds to the position of the posterior mitotic wave of eye development just prior to initiating the culture. Scattered incorporations in the anterior part of the disc derive from the first mitotic wave or to peripodial cell incorporations. The ommatidial clusters (rhodamine-phalloidin: red) that had already formed then (arrowhead), before the culture was initiated, are situated posterior to the band of BrdU labelling. Ommatidial clusters that formed subsequent to this (arrow), during the 10-h culture period, extend anterior to the BrdU front. Overlap between BrdU and phalloidin labelling appears yellow. Extended focus image corresponding to a thickness of 8μm. Anterior lies to the right of the figure. Scale bar 20μm.

Fig. 18. BrdU incorporation of optic lobe cells from late-thrid instar larvae in culture. Dark stained cells are BrdU positive cells, while light pinkish cells are BrdU negative cells counter-stained with neutral red.
Fig. 11. Eye-disc fragment from old pupa (P+24) cultured for 4DIV shows sparse neurite outgrowth (arrow) which lies slightly outside the focal plane. Pigmentation was obvious in the lower part of the explant, but was unevenly distributed. Live culture, photographed under differential interference contrast but through the plastic substrate, not under optimal optical conditions. Scale bar: 20μm.
C. Mitotic Activity \textit{in vitro}

The ages of animals used for establishing primary cultures were younger than P+10, the time at which the second mitotic wave reaches the anterior edge of the eye disc \textit{in vivo} (Campos-Ortega and Hofbauer, 1977), so the continued mitotic activity of eye-discs \textit{in vitro} could therefore be examined by testing for BrdU incorporation in the cultures.

The incorporation of BrdU continued in both cultured eye discs and eye-disc fragments. Eye discs from P+0 that were put immediately into culture medium containing 1\(\mu\)M BrdU for 2h before being fixed, exhibited BrdU incorporation (Fig. 12B, C). A dense band of BrdU incorporation was seen immediately posterior to the morphogenetic furrow in an entire eye disc (Fig. 12B). This wave appeared undisrupted in eye discs that had been sectored (Fig. 12C), indicating that the subdivision of an eye disc did not perturb its pattern of mitoses. Both patterns were qualitatively similar to BrdU incorporation seen \textit{in vivo} (Fig. 12A), and correspond with the second mitotic wave (Ready et al., 1976), because they are both narrow and posterior to the morphogenetic furrow. Scattered BrdU positive cells that were seen posterior to the mitotic wave (Fig. 12B, C). From their position and appearance by reference to their counterparts \textit{in vivo} (Wolff and Ready, 1993), these are thought to be subretinal cells, lying beneath the basal lamina. The first mitotic wave, which is anterior to the furrow, was not obvious: only few dividing cells were seen anterior to the morphogenetic furrow (Fig. 12C).

It is possible that for the first two hours in culture the eye discs still retain factors from the intact animal or a local milieu that is in some way necessary for cell division. In principle, this could explain the similarity between the BrdU incorporation patterns seen in eye-disc cultures and those seen \textit{in vivo}. These factors could then be lost during longer periods \textit{in vitro}. To test this possibility, double pulses of BrdU were applied to see if the mitotic wave moved \textit{in vitro}. Eye discs from late third-instar larvae were exposed to 1\(\mu\)M BrdU from between 0 and 2h, and between 6 and 8h after initiating cultures. This treatment, however, gave only one band of BrdU incorporation close to the furrow, with
Fig. 12. BrdU incorporation in eye-discs. (A) *In vivo* BrdU incorporation in white pupal eye disc. Inset: enlarged area of (A), incorporations among nuclei of the second mitotic wave (arrowhead) posterior to the morphogenetic furrow (arrow) and among nuclei of subretinal cells posterior to the wave (double arrowhead). (B-F) Incorporation in eye discs *in vitro*. (B) Single BrdU pulse applied between 0-2 h in culture to entire white pupal eye disc. Incorporations with a similar distribution to those in (A) are visible. (C) Single BrdU pulse applied between 0-2h in culture to half a white pupal eye disc also incorporates in nuclei with a similar distribution to those in (A). (D) Double BrdU pulse applied between 0-3h and between 6-8h in culture to an entire late third-instar larval eye-disc labels the subretinal cells (arrow) and only one band corresponding to the second mitotic wave (arrowhead). (E-F) Late BrdU pulse applied between 6-8h in culture to entire late third-instar larval eye discs. (E) Only subretinal cell nuclei are labelled (arrowhead). (F) Weak incorporations are also seen in the second mitotic wave (arrowhead). Anterior is to the right of the figure. Scale bar: 50μm, throughout (inset, beneath A: 10μm).
Fig. 12
many BrdU-positive subretinal cells (Fig. 12D). Eye discs from late third-instar larvae cultured for 8h, that were exposed to BrdU during the last two hours, had a band of labeled cells close to the furrow and a few subretinal cells labeled posteriorly (Fig. 12F), suggesting that the culture conditions can support cell division at least up to 6-8 hours in vitro. In a second eye disc, however, only the dividing subretinal cells were labeled (Fig. 12E). The number of such cells incorporating BrdU was moreover reduced in both cases. Possibly a slight age difference existed between the two late third-instar larvae used to establish the cultures shown in Fig. 12E and 12F, and this difference accounted for the different patterns of incorporation of the late pulse.

The incorporation patterns observed under these three experimental procedures were consistent. Up to 40% of discs detached from the coverglass during the immuno-staining procedure so that the number of stained discs was less than the number of discs when cultures were initiated. For single-pulse labeled discs that were put immediately into BrdU an estimated 12 stained preparations (100%) showed a single band of incorporation (Fig. 12B, C). For the double-pulse incorporation experiment an estimated 24 stained preparations (100%) had a single band of incorporation (Fig. 12D). For the single-pulse labeled discs that were cultured for 8h and exposed to BrdU only during the last 2h, an estimated 12 preparations were stained preparations, of which about two-thirds showed no band of incorporation (Fig. 12E) and the remainder had the single band (Fig. 12F), as before. The reason for the inconsistencies of these incorporations from the late pulse may be to do with inaccurate timing of the development in the third-instar larva (see Discussion, Chapter 4).

D. Movement of the Morphogenetic Furrow in Culture

Given that mitotic activity still existed in discs held in culture, it is possible that pattern formation also continued in vitro. This possibility was examined by following the movement of the morphogenetic furrow, because pattern formation arises at the edge of the
furrow (Ready et al. 1976; Wolff and Ready, 1993). The position of the furrow was recorded with respect to a small hole made anterior to the furrow (see 2.2.5A) in an eyedisc from a late larval instar larva (Fig. 13A). The furrow moved past the hole to lie anterior to it after 6.5h in culture (Fig. 13B). Ommatidial clusters appeared posterior to the furrow at all stages, suggesting that along with cell division in the eye disc, the movement of the furrow was accompanied by pattern formation in vitro.

The advance of this area of pattern formation was studied further to indicate the approximate speed of the morphogenetic furrow's movement in vitro. First, the second mitotic wave of eye discs was labeled in early third-instar larvae by in vivo BrdU incorporation (Fig. 14). The discs were then dissected and cultured for 10h. The intense band of BrdU label was positioned with at least five rows of ommatidial clusters anterior to it (Fig. 14), so that there must be at least five rows posterior to the pattern formation front. This indicated that during the culture period pattern formation had advanced for at least five rows, or less than 2h per row.

E. Viability of Cells in Cultured Eye-disc Fragments

Detachment of eye-disc fragments from the substrate was used as a criterion to disqualify culture media. This is because adhesion of disc fragments is the prerequisite for neurite outgrowth, and even for the maintenance of cultures, which if they become detached from the substrate float upward and are decanted off with the next change of the medium. Adhesion cannot reflect whether all individual cells within an attached disc fragment are living, however. For this, calcein AM and ethidium bromide were used to demonstrate cell viability and cell death, respectively, in the cultures.

Calcein AM and ethidium bromide staining indicates that the viability of cells within cultured eye-disc fragments is excellent. For example, a P+5 disc fragment in culture for five days exhibited most cells in a viable state. Viability after longer culture times was not studied. This was indicated by calcein fluorescence of the entire explant, which was
Fig. 13. Movement of furrow from a disc of third-instar larva in vitro. (A) Furrow is located posterior to the marker (arrow), 0h in vitro. (B) Furrow has moved anterior to the marker (arrow), 6h in vitro. Anterior lies to the right of the figure. Scale bar: 50 μm.
sufficiently intense to obscure the outlines of the many individual cells within the explant (Fig. 15A). Very few dead cells were detected by ethidium staining (Fig. 15B). Two reasons may be advanced for the small number of dead cells. First, dead cells or their debris rapidly disappear and the fragment gradually diminishes in size. This seems unlikely, however, because fragment size did not shrink appreciably over a 5-d culture period despite changes in shape. Second, the dead cells seen may reflect the final phase of programmed cell death seen in vivo (Wolff and Ready, 1991). As a result, therefore, the culture conditions adopted here may also have supported the requirements for programmed cell death to occur.

F. Migrating cell fragments (spherules)

For both eye-disc fragments and intact eye disc cultures, small round membrane-bound structures gradually surrounded the culture. Initially the cultures lacked these structures, but gradually after a few hours they were floating in the surrounding medium and later some were settled on the substrate around the culture. They accumulated as the culture continued (Fig. 4). At least some stained with calcein AM, and therefore have esterases (Fig. 15); some were stained with anti-HRP and therefore express a neural antigen. Consistent with these two observations, Hoechst-33258 staining indicated that about 30% of the structures were nucleated, implying that in some cases these structures were cells. Many lacked Hoechst's staining, however, and were thus anucleate. It was not possible to test for the co-localization of stains for nuclei, neural antigens and esterases in the same structures with the methods used in this study. By about three days some of these structures were no longer round, but had become irregular in shape and lacked staining, perhaps because they were debris from cellular structures that had previously migrated from the cultures.

These apparently migrated structures were of varying sizes, ranging from 0.5 μm to 5.0 μm in diameter (Fig. 16). Hoechst-33258 stained nuclei were also of different
Fig. 16. Diameters of migrating spherules and nuclei stained by Hoechst 33258. These ranged from 0.5μm to 5.0μm, with about 50% of the spherules and nuclei having diameters between 1.0-1.5μm. Two cultures, each of about 8 fragments, were used to count spherules and nuclei of the sizes indicated.
sizes. Assigned into groups with 0.5 μm increments in diameter, about 50% of these migrating spherule structures had diameters between 1.0-1.5 μm, and were thus smaller than photoreceptor cells, which had a diameter of about 5 μm. The stained nuclei were fewer but had a similar distribution of sizes (Fig. 16). It seems unlikely that many of the most numerous nuclear size category (1.0-1.5 μm) could have been contained within the most numerous category of spherule, since the latter had the same size. It seems more likely instead that the larger spherules were the nucleated ones. Nuclei in the smallest size category were probably the few that were seriously under the focal plane of the image, and were possibly artifactually small. The real nature of these structures is not known, but some possibilities are discussed below (see Chapter 4).

3.1.3. Cultures of optic lobe cells

A. Optic lobe cell culture

Cultures of optic lobes were established as dissociated cell cultures from the lateral poles of the supraesophageal ganglia of the larval brain.

Cells from late third-instar larvae existed as single cells or as small clusters of cells in culture. Most of the cells survived because they initiated neurite outgrowth within a few hours. The majority (more than 90%) of cells give rise to a single neurite and so appeared as monopolar cells, replicating the majority condition for optic lobe cells in vivo. It was difficult to discriminate between lamina monopolar cells and monopolar cells from the other neuropiles, however, for two reasons: first, it was difficult to dissect the inner from the outer optic lobe anlage (which will be referred to in this section by the name usually given these structures by North American workers, as inner and outer proliferation centres); and second, the outer proliferation centre gives rise to the cellular cortices of both lamina and distal medulla. After five days in culture, the optic lobe cells often differentiated as single cells with bifurcated fibres (Fig. 17A), or as clusters with neurites that fasciculated
Fig. 17. Dissociated optic lobe cells from late third-instar, cultured for 5 days, and stained with anti-HRP immunocytochemistry. (A) A single cell with bifurcated fibers. Varicosities are seen along the neurites. (B). Extending neurites of a cluster of dissociated cells fasciculated together. (C). A single neuron with a big growth cone. Scale bar: 50μm.
Fig. 15. Viability of cells within an eye-disc fragment 7DIV. (A) Calcein AM labelled cytosol of most somata within fragment indicates that its cells are living. Neurites lying beneath the plane of focus were also labelled but are not visible in this micrograph. (B) Occasional nuclei of dead cells labelled by ethidium bromide (arrow). Scale bar: 30μm.
together (Fig. 17B). Arborization patterns could be complex. Some neurites extended up to 100 μm, about the size of the entire optic lobe, thus exceeding the length they would have had in vivo. Neurites from different cell clusters contacted each other to generate apparent networks. Some processes from these neurons had clear growth cones (Fig. 17C) and varicosities (Fig. 17A). Immunoreactivity to anti-HRP indicated that these cells with their extended processes were indeed neuronal in origin (Fig. 17). The specificity of this reaction for a neuronal epitope is based on previous evidence (Jan and Jan, 1982); not all cells are immunoreactive to anti-HRP, those that are not presumably being glial cells. Round cells of two different sizes existed at the early stages after culture initiation. Large cells had a diameter of 10-12 μm and were few in number. They were probably neuroblasts, the stem cells of ganglion cells which have this diameter in the optic lobe (Hofbauer and Campos-Ortega, 1990). The more numerous small cells were 5-7 μm in diameter, and were most likely postmitotic ganglion cells, or their immediate precursors, the ganglion mother cells.

B. Mitotic activity of optic lobe cells in vitro

Optic lobe cells continued their mitotic activity in culture, as did the eye-disc cells (see 3.1.2C). Symmetric and asymmetric cell divisions of large round cells, the presumed neuroblasts, were seen in living optic lobe cell cultures, giving rise to clusters of ganglion cells of smaller sizes. The mitotic cells could be distinguished from the postmitotic cells by BrdU incorporation (Fig. 18). Initial counts of the frequency of BrdU incorporations, however, appeared to declined with the duration in vitro (Fig. 19). Although this temporal pattern correlates with the pattern of mitotic activity in vivo (Hofbauer and Campos-Ortega, 1990), the counts in vitro are based on observations of only two cultures of dissociated cells per sampl time, and were not quantified further.
Fig. 19. BrdU incorporation declines in optic lobe cells as culture continues. Cells from lateral poles of third-instar larval brain have been exposed to 1μM BrdU at different time intervals during the culture period.
II. Eye-disc differentiation and the effects of 20-hydroxyecdysone

As mentioned in the Introduction (Chapter 1, II), 20-HE is an active form of ecdysteroid and ecdysteroids play important roles in nearly all processes in the life-cycle of insects, including moulting, cuticle production and metamorphosis, an influence that is mediated by changes in hormonal titre during these developmental processes (reviewed by Riddiford, 1993). Metamorphosis is of particular interest for studying the effect of ecdysteroids on cell differentiation, because this phase is marked by two changes in the visual system examined here in vitro. These two changes are: 1) the transition from cell proliferation of the imaginal discs during development of the larva to their terminal cell differentiation into adult structures during the pupal stage; and 2) the reorganization of the larval nervous system into a wholly different adult nervous system.

During larval development, the eye imaginal disc is an epithelial sheet initially lacking structural differentiation, from which differentiated photoreceptors of the adult eye form only in the third larval instar, followed by other cell types in the pupa (Wolff and Ready, 1993). The sensitivity of different tissues, or different cell types, towards a particular hormone can often be different. The possibility that ecdysone might differently influence the cellular differentiation of the various cell types in eye-imaginal discs was therefore studied, in addition to its described role in causing disc evagination (Milner and Haynie, 1979). The results indicate that some aspects of differentiation are influenced by ecdysone, while others are not.

3.2.1. Aspects of photoreceptor differentiation that are not influenced by 20-HE

The expression of neuronal antigens and photoreceptor-specific antigens in eye-disc fragments in culture were examined under two culture conditions: one supplementation with 1 µg/ml 20-HE, and the other without the hormone. Immuno-staining for these
antigens in cultures from media lacking 20-HE did not differ visually from immuno-
staining in cultures that had access to 20-HE.

A. Neuronal antigens

The state of neuronal differentiation is preserved in cultures of eye-disc fragments. When cultured \textit{in vitro}, eye-disc fragments from early pupae had extensive fibre
outgrowth. Sometimes the extending processes seemed to be surrounded by epithelial-like
sheets (see Results I; and Li and Meinertzhagen, 1995). To confirm that these fibres were
actual neurites, immunoreactivity to anti-HRP (Jan and Jan, 1982) and to 22C10 (Zipursky
et al., 1984), the two antibodies that label early differentiated neurons in \textit{Drosophila}, was
used to detect the presence of the respective neuronal antigens.

Cultures from P+5 disc fragments expressed antigens to both anti-HRP and 22C10
(Fig. 20 and Fig. 21). After 4DIV, fibres extended vigorously from fragments (Fig.
20A and B). These fibres, together with their cell bodies located within the fragments,
were immunoreactive to anti-HRP antibody (Fig. 20A). The extending neurites
fasciculated and de-fasciculated to form a complicated network among themselves. Anti-
HRP, which recognizes a membrane bound carbohydrate epitope, also stained the
membranes of cell bodies within the fragment proper. These anti-HRP positive cell bodies
co-existed with anti-HRP negative cells, and with non-cellular structures (Fig. 20A).

Surrounding the fragment lay profiles and clusters of profiles, that in some cases were cells
(see Results 3.1.2F; Li and Meinertzhagen, 1995), and that had migrated out from the
fragment, together with cellular debris. Some of the profiles apparently migrated along the
extending neurites. These migrating profiles sometimes themselves sent out processes
which joined at an obtuse angle to the fascicles along which they subsequently traveled
(Fig. 20B). Despite such occasional cases, migrating cells or cell clusters surrounding
the fragment generally failed to extend processes and to fasciculate with those from the
fragment cultures (Fig. 20A).
Fig. 20. Immunoreactivity to anti-HRP of P+5 eye-disc fragments cultured for 4DIV. (A) Structures that were both anti-HRP-positive (arrows) and -negative (arrowheads) were found within, as well as surrounding, the disc fragment. The processes (double arrowhead) from the fragment were anti-HRP positive, but lay at a slightly different focal plane to that of the disc fragment and are photographically under-exposed to reveal the staining of the cell bodies in the explant. (B) Extending neurites formed a complex network. Inset: Neurites enlarged from area enclosed in rectangle in (B), have uneven immunoreactivity along their length, which is exaggerated by the differential interference contrast optics. A migrating profile (open arrow) sent out its own neurites (arrowhead) which fasciculated with the nearby neurite bundle (arrow). Most structures surrounding the fragments lacked neurites. Scale bars (A and B): 20μm; (B) inset: 10μm.
As with anti-HRP immunoreactivity, cell bodies were immunoreactive to the monoclonal antibody 22C10. This antibody also stained the fibres that extended from culture fragments, not only demonstrating that the extending fibres were neurites, but also revealing the relationship of these neurites within the culture fragment itself (Fig. 21A), which could be seen by using the immuno-fluorescence method (section 2.1.4B).

Although the neurites seemed unorganized within the fragment (Fig. 21A), they appeared to be emitted from the fragment at one of only a few sites. Some neurites traveled a long way within the fragment to find this exit, leaving the fragment at its opposite side rather than taking a shorter route to leave at the same side as the cell body (Fig. 21B). Neurites exited the fragment in fascicles of different sizes. Unlike the immunoreactivities detected by anti-HRP (Fig. 20), however, the neurites stained by 22C10 appeared smooth, without spiny branches, and the staining tapered off towards the end of the neurite (Fig. 21A), failing to reveal the growth cone at its very tip that is invariably seen with 24B10 (see below).

B. Photoreceptor antigens

Expression of photoreceptor specific antigen follows the expression of early neuronal antigens (Zipursky et al., 1984). To identify whether neurons in the eye discs that had already expressed immunoreactivity to anti-HRP (Fig. 20) and to antibody 22C10 (Fig. 21), also expressed the antigen chaoptin, which is specific to photoreceptor cells (Van Vactor et al., 1988; Pollock et al., 1990), immunoreactivity to the monoclonal antibody 24B10 (Zipursky et al., 1984, 1985) was also examined.

Cells of eye-disc fragments cultured 3 DIV, from prepupae, were immunoreactive to 24B10, indicating that they had already commenced differentiation into photoreceptors (Fig. 22). The pattern of 24B10 immunoreactivity of disc-fragment cultures was similar to that of 22C10, revealing that the neurites exit the disc fragments as bundles or fascicles (Fig. 22A). Much more detailed features of the extending neurites, now confirmed as
Fig. 21. 22C10 immunoreactivity of P+5 eye-disc fragments cultured 3DIV. (A) All cell bodies were stained, but most were not in the focal plane of the micrograph, except one (arrow head) at the periphery of the fragment. Neurites were also stained. A cluster of cells (arrow) extended its own neurites to join a nearby neurite fascicle. Growth cones were not immunoreactive to 22C10. (B) Neurites of a cell cluster passed the exit site (arrowhead) closest to its cell bodies to travel by a longer path, in leaving the fragment by the opposite side of the fragment (arrow). Scale bars (A): 40μm; (B): 20μm.
Fig. 22. 24B10 immunoreactivity of P+5 eye-disc fragments cultured 3DIV.  
(A) Photoreceptor axons emerging from eye-disc fragment as big bundles. Growth cones (arrowhead) and filopodia are clearly immunoreactive to 24B10. Two immuno-positive profiles migrated along an axon fascicle (arrow). Cell bodies within the fragment lie out of focus. For double arrowhead, see legend to Fig. 6. (B) Membranes of photoreceptor clusters within the fragment are clearly labelled by 24B10. Scale bars (A): 40μm; (B): 20μm.
Fig. 22
photoreceptor axons, were revealed by 24B10 than by 22C10 immuno-staining, however. Growth cones and fine filopodia at the tip of the neurites, which were not seen with 22C10 staining, were clearly demonstrated by 24B10 immunofluorescent staining (Fig. 22A). This is not because of a difference in the intensity of fluorescence, as judged by eye. The neurites seemed less smooth after 24B10 staining than with 22C10 staining, having swellings and fine spines along the length of the extending axons (Fig. 22A). The membrane of the photoreceptor cell bodies were intensely stained by 24B10, revealing groups of photoreceptor cell bodies resembling ommatidial clusters (Fig. 22B). A few immuno-positive profiles, photoreceptor cells or their fragments, migrated from the disc fragment, and these occasionally sent out their neurites either to contact or to join the fascicles of nearby extending neurites. Immunoreactivity to 24B10 demonstrated that the early differentiation of neurons, but now identified as photoreceptors, was maintained in disc fragment cultures. Optic lobe cells do not express 24B10 immunoreactivity.

C. Neurotransmitter differentiation

Following from evidence in larger fly species (Hardie, 1987, 1988), there is now considerable evidence that the neurotransmitter for all imaginal photoreceptors in Drosophila is histamine (Pollack and Hofbauer, 1991; Sarthy, 1991). Because cultured eye-disc fragments exhibited early signs of photoreceptor differentiation, it seemed possible that they might also synthesize histamine.

The exact time of onset for in vivo histamine synthesis in eye discs is not yet known. Histamine-like immunoreactivity has been detected here in photoreceptors of eyes from the 50% pupal development stage (Fig. 23B) and from pharate adults (at about four days after pupariation) (Fig. 23A). Immunoreactivity in vivo was detected in photoreceptor cell bodies, in their axons and especially in their terminals in the lamina and medulla (Fig. 23A), as in mature adult Drosophila (Pollack and Hofbauer, 1991). In
Fig. 23. Histamine immunoreactivity in preparations *in vivo* (A, B) and *in vitro* (C, D).

(A) Pharate adult, \( \text{h} \) immunoreactivity in terminals of R1-R6 in the lamina (arrow) and R7, R8 in the distal medulla (arrowhead), as well as in the lobula (double arrowheads).

(B) Pupa at about 50% of development, with immunoreactivity in the lamina derived from terminals of R1-R6 (arrowhead) which are still shallow at this stage. The retina (arrow), identifiable by corneal facets, is still a slender sheet, deformed in shape during preparation. Immunoreactivity is not apparent in the medulla at this stage. (C) P+5 eye-disc fragment cultured 5DIV. Photoreceptor axons exhibit faint immunoreactivity (arrow). Compare staining with control, a P+5 eye-disc fragment at 5DIV in which primary antibody was omitted during immunostaining (D). Scale bars: 40\( \mu \text{m} \).
Fig. 23. Histamine immunoreactivity in preparations in vivo (A, B) and in vitro (C, D).

(A) Pharate adult, with immunoreactivity in terminals of R1-R6 in the lamina (arrow) and R7, R8 in the distal medulla (arrowhead), as well as in the lobula (double arrowheads).

(B) Pupa at about 50% of development, with immunoreactivity in the lamina derived from terminals of R1-R6 (arrowhead) which are still shallow at this stage. The retina (arrow), identifiable by corneal facets, is still a slender sheet, deformed in shape during preparation. Immunoreactivity is not apparent in the medulla at this stage.

(C) P+5 eye-disc fragment cultured 5DIV. Photoreceptor axons exhibit faint immunoreactivity (arrow). Compare staining with control, a P+5 eye-disc fragment at 5DIV in which primary antibody was omitted during immunostaining (D). Scale bars: 40μm.
addition, immunoreactivity was seen in one of the third neuropils, the lobula (Fig. 23A), which was obviously not of photoreceptor origin.

Histamine-like immunoreactivity was also detected in cultures of eye-disc fragments held in vitro. Cultures from P+5 showed anti-histamine immunoreactivity after 5DIV, detected using the immunofluorescence method. The cell bodies exhibited fluorescence (Fig. 23C) but this was indistinguishable from that seen in cultures stained by control immunocytochemical procedures (Fig. 23D). Immunoreactivity was also seen along the extending photoreceptor axons (Fig. 23C) which, unlike their cell bodies which are located within the fragment proper, however, was not seen in control stains (Fig. 23D). Just as for the pattern seen with 24B10, fine spines and growth cones were clearly immunoreactive to anti-histamine (Fig. 23C). The two patterns differed, on the other hand, because immunoreactivity to the mouse monoclonal antibody 24B10 was expressed over the membranes whereas histamine-like immunoreactivity to the rabbit polyclonal antibody was presumably cytosolic or vesicular in origin.

In double-stained preparations, the anti-histamine image of photoreceptor axon fascicles was wider than the same image stained by 22C10 (Fig. 24). The cell profiles for both immunoreactivities lay in the same focal plane, so this cannot account for the different widths of the two images. Possibly the difference was caused by the envelopment of fascicles by lamellae of non-neuronal origin (see later, Fig. 31). These lamellae were perhaps glia that were capable of the re-uptake of histamine that had been released from photoreceptor neurites. This result was the same regardless of whether cultures were grown in medium lacking 20-HE or in 20-HE supplemented medium.
3.2.2. Aspects of differentiation of disc cells that are influenced by 20-HE

A. Expression of synaptotagmin

In view of the presence of histamine in differentiating eye disc fragments (above), the expression of a synaptic vesicle protein, synaptotagmin, was also tested in eye-disc fragment cultures, to see if the present culture conditions could support the differentiation of an important component of the machinery for release of transmitter (Littleton et al., 1993a,b; DiAntonio and Schwarz, 1994), as well as to test a marker for synaptic vesicles.

Synaptotagmin was expressed in eye disc fragments in vitro. A 3-day old culture from P+5 (the same as shown in Fig. 22A) was double stained with 24B10 and DSYT2, a polyclonal antibody for Drosophila synaptotagmin (Littleton et al., 1993a). Punctate immunoreactivity to DSYT2 was detected along the extending photoreceptor axons (Fig. 25), suggesting the presence of synaptotagmin. The distribution of synaptotagmin immunoreactivity was not even. Some parts of the axons lacked staining to DSYT2, while most of the axonal terminals and growth cones were strongly immunoreactive. Cell bodies of the photoreceptors were not stained, giving the disc a ghost-like appearance with a number of DSYT2-positive fibres extending from it (Fig. 25). From its distribution, the DSYT2-immunoreactivity appeared to be cytosolic (Fig. 25) compared with the appearance of the presumed plasmalemma staining of 24B10 (Fig. 22A). The pattern of DSYT2 immunoreactivity in culture suggests that synaptotagmin was present in photoreceptor neurites. Given the normal localization on synaptic vesicles of the vertebrate homologue to the antigen (Bennett et al., 1992), DSYT2 immunoreactivity perhaps implies that synaptic vesicles formed in vitro in photoreceptor axons. Moreover, incidentally, these axons lacked their normal target neurons, the lamina monopolar neurons.

The expression of synaptotagmin was influenced by 20-HE. The immunoreactivity of 24B10 and synaptotagmin immunoreactivity were compared in cultures of disc fragments treated with 1μg/ml 20-HE (Fig. 26A and B) with those
addition, immunoreactivity was seen in one of the third neuropils, the lobula (Fig. 23A), which was obviously not of photoreceptor origin.

Histamine-like immunoreactivity was also detected in cultures of eye-disc fragments held \textit{in vitro}. Cultures from P+5 showed anti-histamine immunoreactivity after 5DIV, detected using the immunofluorescence method. The cell bodies exhibited fluorescence (Fig. 23C) but this was indistinguishable from that seen in cultures stained by control immunocytochemical procedures (Fig. 23D). Immunoreactivity was also seen along the extending photoreceptor axons (Fig. 23C) which, unlike their cell bodies which are located within the fragment proper, however, was not seen in control stains (Fig. 23D). Just as for the pattern seen with 24B10, fine spines and growth cones were clearly immunoreactive to anti-histamine (Fig. 23C). The two patterns differed, on the other hand, because immunoreactivity to the mouse monoclonal antibody 24B10 was expressed over the membranes whereas histamine-like immunoreactivity to the rabbit polyclonal antibody was presumably cytosolic or vesicular in origin.

In double-stained preparations, the anti-histamine image of photoreceptor axon fascicles was wider than the same image stained by 22C10 (Fig. 24). The cell profiles for both immunoreactivities lay in the same focal plane, so this cannot account for the different widths of the two images. Possibly the difference was caused by the envelopment of fascicles by lamellae of non-neuronal origin (see later, Fig. 31). These lamellae were perhaps glia that were capable of the re-uptake of histamine that had been released from photoreceptor neurites. This result was the same regardless of whether cultures were grown in medium lacking 20-HE or in 20-HE supplemented medium.
Fig. 24. Double staining of eye disc fragment with 22C10 (A) and anti-histamine (B).

Immunoreactivity to 22C10 is more intense and limited to neurites, whereas to anti-histamine is fainter and not restricted to the neurites, which are contained within broader profiles of staining (compare the same neurite in A and B, arrowheads). Scale bar: 40 µm.
Fig. 25. DSYT2 immunoreactivity in the same P+5 eye-disc fragment cultured 3DIV as in Fig. 22A. Synaptotagmin is expressed in a punctate pattern along most neurites visible in Fig. 22A by 24B10 immunoreactivity. Compare the neurite indicated by double arrowheads in both figures. Cell bodies lack DSYT2 immunoreactivity, their neurites visible crossing the disc fragment (arrow). Scale bar: 40 μm.
Fig. 26. Double immunoreactivities of 24B10 (A, C) and DSYT2 (B, D) in P+5 eye-disc fragments after 3DIV. The eye-disc fragments were cultured in the presence (A, B) or absence (C, D) of 1μg/ml 20-HE. There was no visible difference between the two culture conditions for 24B10 immunoreactivity (A, C). In the case of DSYT2 immunoreactivity there were more spots along the neurites in the presence of 1μg/ml 20-HE (B) than in its absence (D). In both cases, most growth cones contained DSYT2 immunoreactivity (arrow). Scale bars: 20μm.
cultured without 20-HE (Fig. 26C and D). Although the intensity of 24B10 immunoreactivity seemed the same under both conditions, the number of DSYT2-immunoreactive spots along the length of the neurites appeared to be larger in fragments cultured in the presence of 20-HE (Fig. 26B) than those in the absence of 20-HE (Fig. 26D). This impression was gained by examining at least four explants in each of more than five cultures, but could not be quantified, however, because the sizes of immunoreactive spots were extremely uneven in individual neurites. Despite these apparent differences in their number, the distribution of spots under the two conditions (with or without 20-HE) seemed, on the other hand, not to differ.

B. Effect of 20-HE on neurite outgrowth

The lengths of neurites of photoreceptors were greatly increased in cultures exposed to 20-HE. Eye-disc fragments from P+5 were cultured in media with or without supplementation with 1μg/ml 20-HE. Under both conditions, disc fragments had extensive outgrowth of photoreceptor axons, with the axons from the same fragment exhibiting a large range of lengths. The neurite lengths from disc fragments were therefore averaged. In cultures supplemented with 20-HE, the average neurite length was significantly greater than the average in cultures lacking supplementation with 20-HE. This difference was detected as early as 2DIV and persisted up to at least 6DIV (Fig. 27A). Both the length and number of individual axons increased with the duration of the culture, under both culture conditions. For each condition, the neurites of at least 10 fragments in a single culture were measured, with the number of fragments being the sample number used for statistical comparison. For the particular cultures tested, the length increase was greater than the increase in neurite number, for the first four days, resulting in an increase in average neurite length. This increase was only temporary, however. On the fifth day, the number of short, newly extended neurites increased, decreasing the average neurite length.
Even so, average neurite length in cultures exposed to 20-HE was still greater than that in cultures lacking 20-HE, even after 5DIV.

Even though the numbers of neurites in cultures supplemented with 20-HE (Fig. 26A) seemed greater than those of cultures lacking exposure to 20-HE (Fig. 26C), the different sizes of disc fragments, and the different numbers of cells contained in each fragment, meant that it was not possible to make such comparisons accurately.

C. The effects of different 20-HE concentrations

In order to quantify further the effects of 20-HE, neurite outgrowth was measured after eye-disc fragments from P+3 had been cultured 5DIV in the presence of various concentrations of 20-HE. This made it possible to examine the dose-response curve for neurite outgrowth, a marker which is more readily quantifiable than alternative aspects of neuronal differentiation that relied upon immunoreactive detection. The concentrations were examined in an initial series of 0, 0.5, 1.0 and 2.0 µg/ml 20-HE. All cultures containing 20-HE had on average significantly longer neurites than those from disc fragments cultured in a medium lacking 20-HE (Fig. 27B). Moreover, neurite length was not further increased in the presence of 20-HE concentrations greater than 0.5 µg/ml. Differences in neurite length among cultures in 0.5, 1.0 and 2.0 µg/ml 20-HE were not statistically significant (Fig. 27B). This result suggested that the 1.0µg/ml of 20-HE used in the present culture condition should exert the maximum effect of this hormone on neurite outgrowth.

In order to try to refine further the dose-response curve for neurite outgrowth and determine the threshold for 20-HE action, neurite outgrowth was also examined at lower concentrations of 0.125, 0.25 and 0.5 µg/ml 20-HE (Fig. 27B). Average neurite lengths were 120, 105 and 103 µm respectively. All values again differed from that of the control neurites, which was 71µm, but again did not significantly differ from each other. These results thus fail to demonstrate the dose-response function for the action of 20-HE on
Fig. 27. (A) Effects of 20-HE on the average neurite length of photoreceptor axons in vitro. The average length (± SD) is greater in cultures exposed to 1μg/ml 20-HE than that in cultures lacking 1μg/ml 20-HE. The difference is statistically significant throughout 2DIV to 6DIV. * is p < 0.05. Numbers above bars are numbers of culture fragments.
Fig. 27. (B) Dose-response curves of 20-HE on average neurite length (± SD). Cultures of low concentration (0-0.5 mg/ml 20-HE) were 4DIV, and cultures of higher concentration (0-2.0 mg/ml 20-HE) were 5DIV. Neurite length was greater in cultures treated with any of the tested 20-HE concentrations than in cultures lacking 20-HE. This difference was statistically significant. No statistically significant difference existed among neurite lengths of cultures in different 20-HE concentrations. Numbers above bars are numbers of culture fragments.
neurite length, for which the unsaturated part of the curve lies at doses lower than at 0.125 μg/ml.

D. Differentiation of non-neuronal cells

Pigment cell differentiation. There are two classes of screening pigments contained in pigment cells: the brown ommochrome pigments and the red drosoptrines, and the first appearance of each of these differs in vivo. Ommochrome pigmentation starts around 48 hours after pupariation, while the drosoptrines can usually be detected about 96 hours after pupariation (Shoup, 1966). To examine whether the pigmentation detected in eye-discs in vitro (Result I, 3.1.2B) represents the differentiation of both brown and red pigments, eye colour mutants were used which lack one or other of the two pigmented systems, to study pigmentation in vitro. The two mutants used were brown (bw), which fails to produce red pigments, the drosoptrines, and vermilion (v), which does not form brown pigments, the ommochromes (Lindsley and Zimm, 1992).

The formation of ommochromes in bw mutant and wild type (wt) eye-disc cultures both required the presence of 20-HE. Eye-discs from P+5 of wt and bw were cultured in media supplemented with 1 μg/ml 20-HE (Fig. 28A, C, E) or in a medium without such supplementation (Fig. 28B, D, F). With 20-HE supplementation, cultures of both wt (Fig. 28A) and bw (Fig. 28C) started to show pigmentation at about 2DIV, similar to the time at which pigmentation can be detected in vivo in wt and bw pupae. No sign of pigmentation was seen in cultures lacking 20-HE supplementation (Fig. 28B, D, F).

The formation of drosoptrines, by contrast, could not be detected under any condition in vitro. Eye-disc cultures of v (Fig. 28E, F) failed to synthesize pigment, even in the presence of 1 μg/ml 20-HE (Fig. 28E). Addition to the medium of 0.1 mg/ml guanine, the substrate for drosoptrine synthesis (Sullivan and Sullivan, 1975), also did not initiate pigmentation. One possibility for this failure might have been that, in addition to the substrate guanine, other factors, which are normally present in vivo in the fly and are
Fig. 28. Eye disc pigmentation in vitro. Eye disc fragments cultured in the presence (A, C, E) or absence (B, D, F) of 1 µg/ml 20-HE. (A, B) Wild-type disc fragments. (C, D) Disc fragments of brown mutant. (E, F) Disc fragments of vermillion mutant. Different focal planes were used to demonstrate the pigmentation of disc fragments. Neurites were in focal plane in (A), (B) and (C), but not in focal plane in (D), (E) and (F). Scale bars: 20µm.

Fig. 30. Lens formation in a P+5 eye-disc fragment cultured 3DIV in the presence of 1µg/ml 20-HE. (A) Disc fragment that had sealed in culture to form a partial un-everted vesicle with the apical surface facing internally. Structures that were lens-shaped in profile (arrowheads) were seen on the apical surface of the disc fragment, which after epifluorescent illumination (B) exhibited autofluorescence typical of sections of adult cornea. Scale bars: 10µm.
Fig. 28+30
crucial for either guanine uptake or for drosopterine synthesis, were missing in my culture conditions. The cultures were therefore examined in a medium which had been supplemented with a fly extract (see Chapter 2, 2.3.5) at 50μl/ml. Pigmentation was still not detected in eye-discs from v, however, even after culturing under this condition.

**Bristle and lens differentiation.** The differentiation of mechanosensory bristles in eye-disc cultures required the presence of 20-HE. Eye-disc fragments from P+5 cultured for three days in a medium supplemented with 1μg/ml 20-HE generally exhibited the differentiation of bristles. As an example, one fragment, which formed the shape of a vesicle, had evenly spaced bristles clearly visible using differential interference contrast optics (Fig. 29). The cell divisions generating the bristle cells normally start *in vivo* at approximately 14 hours after pupariation (Wolff and Ready, 1993), and as our cultures were initiated at about 5 hours after pupariation, bristle cells seen in cultures must have been generated *in vitro*. This evidence further indicates that mitotic activity continued under the culture conditions used. In contrast, bristles could not be detected in cultures which lacked 20-HE supplementation. Whether bristle formation in cultures lacking 20-HE was blocked at the level of cell divisions or at the level of the final secretion of a bristle is not clear, however.

Secretion of the corneal lens in eye-disc cultures is also greatly influenced by the presence of 20-HE. Lens-shaped structures could frequently be found at the surface of eye-disc fragments from P+5 onwards, when eye disc fragments were cultured in a medium containing 1μg/ml 20-HE (Fig. 30A, B), whereas similar structures were absent in disc fragments cultured in a medium devoid of 20-HE. The culture illustrated in Fig. 30 had sealed into the shape of a vesicle. The lens of the adult ommatidium normally exhibit auto-fluorescence after exposure to UV light. The disc fragment cultured in the presence of 20-HE had a patterned array of similar autofluorescence, which together with
Fig. 29. Interommatidial bristle formation in vitro. The P+5 eye-disc fragment was cultured 3DIV in a medium containing 1µg/ml 20-HE. Regularly spaced bristles (arrowheads) formed in culture. Extending photoreceptor neurites that also formed in this culture fell outside the focal plane of the bristles. Scale bar: 20µm.
the shape of the structures, suggested that corneal lenses had formed in the cultures (Fig. 30B). Auto-fluorescence was not detected in cultures lacking 20-HE. This observation suggests that without 20-HE lens formation was either stopped or delayed, and that lens secretion consequently requires 20-HE.

_Glial-like envelopment of neurites._ In cultures containing neurite outgrowth, photoreceptor axons were surrounded by a thin envelopment of membrane. This sheet (Fig. 31) was not detected with anti-HRP (Fig. 20) and is thus non-neuronal. The cellular identity of the sheets is otherwise not clear. Neither cell bodies nor nuclei are apparent. These structures were present from the earliest times of culture and invariably accompanied by photoreceptor neurites (see Results I, 3.1.2A; and Li and Meinertzhagen, 1995). Structures like these enwrap the neurite fascicles down almost to the growth cone tip (Fig. 31), where they flatten out and extend laterally from the neurite shaft for distances up to several microns between individual defasciculated neurites. Although these structures occurred in cultures that lacked 20-HE (Fig. 31C, D), they were also found in cultures supplemented with 1μg/ml 20-HE (Fig. 31A, B), but under these conditions their flattening was less pronounced. This difference is clearly seen between the flattened profile in Figure 31D and the clean-cut edge of neurites in Figure 31B. These examples were representative of the neurites examined under both culture conditions in at least three explants, in each of three cultures that were examined by SEM.
Fig. 31. SEM of non-neuronal sheet enveloping neurites from P+5 eye-disc fragment cultures at 3DIV in the presence (A, B) or absence (C, D) of 1μg/ml 20-HE. Large fascicles extended from the eye-disc fragments were wrapped by non-neuronal sheets under both culture conditions (A, C). The non-neuronal sheets were more obvious at the neurite terminals when 20-HE was lacking (D) than for neurites that were exposed to 20-HE at 1μg/ml (B). Scale bars: (A, C) 10μm; (B, D) 5μm.
III. The regeneration of photoreceptor axons in vitro and their interaction with optic lobe cell

Results I and II have demonstrated that the culture conditions used can support the extension of photoreceptor axons from larval and prepupal eye-imaginal discs. Whether these neurites are extending neurites of ommatidia, which are newly formed in vitro, or whether they are regenerated from the severed neurites of pre-existing ommatidia, is however still unclear. The results presented in this section provide direct evidence that severed photoreceptor axons are able to regenerate in culture, and that the regenerated axons that arise from such stumps have the potential to interact with their target neurons.

3.3.1. Regeneration of photoreceptor axons in vitro

To examine the regeneration of photoreceptor axons, low concentrations (0.05%) of the protease trypsin were used to dissociate partially the eye-disc fragments. This treatment exposed some individual ommatidial clusters with severed axonal stumps, and allowed direct observation of these axonal stumps during the period of the culture.

Immediately after initiating a representative culture, an ommatidial cluster with its axonal stump from a P+5 fragment was floating in the culture medium. After two hours, the stump had settled onto the culture substrate (Fig. 32A) and started to elongate. Two hours later neurite length was obviously greater (Fig. 32B) compared with Fig. 32A. A single dissociated cell associated with the same disc fragment also had a severed axonal stump which attached to the substrate (Fig. 32A), and elongated during the culture period (Fig. 32B). The growth rate of neurites after initiating the culture was not constant. Generally the rate was fast immediately after culture initiation, and slowed down as culture continued. Eventually the extending neurites stopped growing when they reached their maximum length. The growth rate of the two regenerating neurites in Fig. 32 were about 5.5 and 7.5 μm/hr respectively, reflecting the initial fast growth phase of neurites.
Fig. 32. Regeneration of severed photoreceptor-axons *in vitro*. Eye-disc fragments from P+5 were slightly enzymatically treated. After 2h of culture initiation, two floating axonal stumps, one from the fragment (arrow) and the other from a dissociated cell (arrowhead), has neattached to the culture surface (A). During the next 2h, these two axonal stumps has increased in length (B). Scale bar: 20μm.
elsewhere. Growth cones were not obvious at the magnification that was used to record growth, because the long-working distance objective (16X/0.35) used to observe living cultures had insufficient numerical aperture to resolve such small organelles. These observations indicated that the cut ends of photoreceptor axons from P+5 eye discs had the ability to regenerate.

It will be demonstrated later, for reasons to be discussed in Chapter 4, that most of the extending neurites seen in the cultures used in this study were in fact regenerating photoreceptor axons, rather than photoreceptors that differentiated de novo, even if the culture conditions used in this study did in fact support both forms of photoreceptor growth.

3.3.2. Influence of histamine on photoreceptor outgrowth

Neurotransmitters, in addition to their role as chemical mediators for neurotransmission in the adult nervous system, have also been shown to have regulatory functions during neural development (Lipton and Kater, 1989; reviewed by Lauder, 1993). For example, serotonin limits the outgrowth of neurites from serotoninergic neurons in vitro, an autoregulatory action that may be interpreted as the ability to restrict the growth of its neurites by the local release of its own transmitter. Autoregulatory functions of serotonin on neurite growth have been demonstrated both in vivo using Drosophila mutants incapable of serotonin synthesis (Budnik et al., 1989) and in the gastropod Helisoma, both in vivo (Goldberg and Kater, 1989) and in vitro with cell cultures (Goldberg et al., 1991). Given that cultured eye-disc fragments have demonstrated the synthesis of histamine-like immunoreactivity in vitro, the possibility was therefore examined that histamine might also have an autoregulatory effect, causing either enhancement or inhibition of neurites outgrowth among regenerating photoreceptor axons in culture.

Eye-disc fragments from P+5 were cultured in media containing 0, 0.1, 0.5, and 1.0 mM histamine for two culture periods: 4DIV and 6DIV. For both culture periods, the
presence of histamine at 0.1 mM seemed to increase the average neurite length slightly, compared with controls remaining in a medium that lacked histamine (Fig. 33). These differences could be compared using a t-test because each comparison (between average neurite lengths at 0 and at a particular histamine concentration) was independent of the other comparisons. The differences (for both culture periods) were, however, not statistically significant (Student t-test). On the other hand, histamine concentrations higher than 0.1 mM did alter the average neurite length, reducing this for cultures held both 4DIV or 6DIV. Only in one group, however, were these decreases significant. This was for the cultures exposed to 1.0 mM histamine for 6DIV, in which the average neurite length was significantly shorter (by 27.7%) than in controls (Fig. 33). These tests would not have revealed the possible existence of trends between different histamine concentrations, for which an ANOVA test would have been necessary.

3.3.3. Possible interactions between regenerated photoreceptor axons and their potential postsynaptic neurons

Are regenerated photoreceptor axons capable of synaptic regeneration? Given that the likely photoreceptor neurotransmitter, histamine (Pollack and Hofbauer, 1991; Sarthy, 1991), as well as the synaptic vesicle protein, synaptotagmin, have both been detected immunocytochemically in the axons of cultured eye-disc fragment, the predominantly regenerated photoreceptor axons might be able to form functional synapses if the appropriate target neurons, the lamina monopolar cells, were also present. This possibility was examined by co-culturing eye-disc fragments with dissociated optic lobe cells from the lateral poles of prepupal or larval brains. (As mentioned in the Introduction (Chapter 1), the precursor cells for lamina formation reside in the outer proliferation centre, at the lateral pole of the larval brain).

Eye-disc fragments and dissociated optic lobe cells from P+5 were co-cultured 4DIV. Neurites extended from both cell populations. Sometimes the neurites from optic
Fig. 33. Effects of histamine on average neurite length of photoreceptor axons (± SD). Cultures were maintained for 4DIV and 6DIV with a histamine concentration series of 0, 0.1, 0.5 and 1.0 mM. No statistical difference has been found in cultures of 4DIV between neurite length of any of concentration tested and the control (0 mM). A significant decrease of neurite length was only detected after 6DIV in culture with 1.0 mM histamine. Numbers above bars are numbers of culture fragments. * is p ≤ 0.05.
lobe cells contacted neurites from eye-disc fragments, or *vice versa* (Fig. 34). It was hard
to test whether the interactions were specific or not, however, because of the lack of
markers for the optic lobe cells. SEM of a co-culture did however demonstrate neurite
interactions between the two cell populations (Fig. 35). The neurites from the optic lobe
cells could be distinguished from those belonging to photoreceptors, because the optic lobe
cells had no corneal-like secretion covering the eye-disc fragment as the photoreceptors did,
and were more completely dissociated. These LM and SEM observations immediately
suggested the possibility that regenerating photoreceptor axons could interact with optic
lobe cells with which they were co-cultured.

To examine the possibility that synaptic contacts form between the two cell
populations, TEM was used to reveal the ultrastructure of points of contact in the 6DIV co-
cultures. More than two synapse-like structures were found in one co-culture examined
from P+5 (Fig. 36). At the site of synaptic-like contact, the two opposing membranes of
the two neurite terminals were parallel to each other. There was a widened space 27 nm
between the two membrane, resembling the synaptic cleft. The putative presynaptic
membrane had a density at the site of the cleft which extended 0.29 μm (Fig. 36, inset).
Synaptic vesicle-like structures were seen at the terminal of neurites and around the putative
synapse (Fig. 36). The size of these vesicles was estimated to be about 70 nm in
diameter. Bundles of microtubules were seen in neighbouring neurite processes, with
some associated with membrane bound vesicles (Fig. 36). Fewer microtubules were
seen around the synaptic-like structures. Electron micrographs that could have revealed the
glial envelopment surrounding free photoreceptor neurites were not examined, so that the
existence of such glia in the co-cultures could not be demonstrated.

Several discrepancies existed between the structure of these putative synaptic
contacts seen in the co-culture and the well established mature tetrad synapses seen *in vivo*
in the corresponding lamina of flies (Fröhlich and Meinertzhagen, 1982; Fröhlich, 1987;
Meinertzhagen and O'Neil, 1991). Firstly, the presynaptic ribbon with its platform, a
3.2.2. Aspects of differentiation of disc cells that are influenced by 20-HE

A. Expression of synaptotagmin

In view of the presence of histamine in differentiating eye disc fragments (above), the expression of a synaptic vesicle protein, synaptotagmin, was also tested in eye-disc fragment cultures, to see if the present culture conditions could support the differentiation of an important component of the machinery for release of transmitter (Littleton et al., 1993a,b; DiAntonio and Schwarz, 1994), as well as to test a marker for synaptic vesicles.

Synaptotagmin was expressed in eye disc fragments in vitro. A 3-day old culture from P+5 (the same as shown in Fig. 22A) was double stained with 24B10 and DSYT2, a polyclonal antibody for Drosophila synaptotagmin (Littleton et al., 1993a). Punctate immunoreactivity to DSYT2 was detected along the extending photoreceptor axons (Fig. 25), suggesting the presence of synaptotagmin. The distribution of synaptotagmin immunoreactivity was not even. Some parts of the axons lacked staining to DSYT2, while most of the axonal terminals and growth cones were strongly immunoreactive. Cell bodies of the photoreceptors were not stained, giving the disc proper a ghost-like appearance with a number of DSYT2-positive fibres extending from it (Fig. 25). From its distribution, the DSYT2-immunoreactivity appeared to be cytosolic (Fig. 25) compared with the appearance of the presumed plasmalemma staining of 24B10 (Fig. 22A). The pattern of DSYT2 immunoreactivity in culture suggests that synaptotagmin was present in photoreceptor neurites. Given the normal localization on synaptic vesicles of the vertebrate homologue to the antigen (Bennett et al., 1992), DSYT2 immunoreactivity perhaps implies that synaptic vesicles formed in vitro in photoreceptor axons. Moreover, incidentally, these axons lacked their normal target neurons, the lamina monopolar neurons.

The expression of synaptotagmin was influenced by 20-HE. The immunoreactivity of 24B10 and synaptotagmin immunoreactivity were compared in cultures of disc fragments treated with 1μg/ml 20-HE (Fig. 26A and B) with those
Fig. 34. Co-culture of eye-disc fragments and dissociated optic cells. An optic lobe cell sends out its neurite to contact the photoreceptor axonal fascicle. The contact site is shown in higher magnification in inset. Scale bars: 10μm.
similar acetylation and oxidation systems to those in the locust, to inactivate histamine. Whether the enzymes for inactivating histamine exist in glial cells or not is not yet known.

The presence of histamine immunoreactivity in profiles that are wider than the width of the neurites proper, combined with the SEM observation of enwrapping of neurites by non-neuronal sheets, apparently glial, together suggest that this immunoreactivity is localized not only in the neurite but also in these sheets. I interpret this immunoreactivity as arising from glial re-uptake of histamine that had been released in vitro from photoreceptor neurites (see Chapter 3). The uptake of histamine was not seen in vivo in the neuropiles of the locust optic lobe, however (Elias and Evans, 1984). The finding in this Thesis, that the presumed glia that exist in vitro as thin sheets, and that are inferred to be histamine-immunoreactive, but which in vitro are free to spread out around the growing neurite and thus are visible by light microscopy, is compatible with a suggestion made by Elias and Evans (1983). These authors speculate that the thin glial sheets that exist in vivo, and that surround the neuronal elements of the neuropile, are responsible for uptake of histamine, but that they are too thin in vivo to be visible at the light microscope level. Only the expanded parts of these cells, in the region medial to the medulla, they claim, are visible, so as to explain the localization of histamine uptake to the area other than the neuropile proper. The in vivo structural organization of glia in the fly's lamina is similar to that proposed for the locust's visual system, with lamina epithelial glial cells surrounding the synaptic terminals of photoreceptors (Trujillo-Cenóz, 1965; Meinertzhagen and O'Neil, 1991). Järvilehto and Harjula (1993) indeed find histamine immunoreactivity in glial cells in the lamina of the blowfly Calliphora, compatible with an active role for these cells in the re-uptake of transmitter.

4.2.5. Expression of synaptotagmin

Synaptotagmin is an important protein of the synaptic vesicle membrane, crucially involved in calcium-activated neurotransmitter release in Drosophila (Littleton et al.,
Fig. 35. SEM of co-cultured eye-disc fragment (arrow) and dissociated optic lobe cells (arrowhead). Neurites are seen to extend from both cell populations and contact each other. The apparently directed outgrowth of neurites between eye-disc fragment and optic lobe cells in this example is not seen all cultures. Cornea-like secretion is seen on top of the eye-disc fragment. Scale bar: 50µm.
Fig. 36. TEM of eye-disc and optic lobe cell co-cultures. Abundant microtubules (arrowhead) are present in the neurites. A synaptic like structure (arrow) is seen with an electron-dense region and with membranous vesicular structures. Inset: this region is enlarged to demonstrate the detailed structure, especially the presence of vesicles (arrowhead). Scale bar: 0.5μm, inset: 0.2μm.
Chapter 4
DISCUSSION

I. Culture conditions

4.1.1. Cultures of eye discs

Of the five culture media tested, the best survival and neurite outgrowth from eye imaginal discs were obtained using MM3 medium supplemented with 2% FBS. This culture medium and the original Shields and Sang's M3 medium from which it derives have been used successfully for in vitro studies of imaginal disc differentiation, such as the morphogenetic fusion of eye-antennal discs (Milner and Haynie, 1979, Milner, 1980) and the differentiation of leg and wing imaginal discs (Currie et al., 1988), as well as the establishment of cell lines from these discs (Currie et al., 1988, Cullen and Milner, 1991). Although occasional neurite outgrowth from eye discs has been seen previously (Milner, 1980), consistent survival and neurite outgrowth from eye imaginal discs have not previously been reported. By removing the peripodial membrane and parting the eye discs into fragments, it became possible to obtain repeatable, vigorous neurite outgrowth and satisfactory eye disc survival. This repeatability is essential for the valid comparison of retinal cultures that have received different treatments (see Chapter 3, Results II).

The long-term survival of photoreceptor cells in vivo requires their connection with the lamina, as indicated by a study on the mutant disconnected (Campos et al., 1992). Without connection to the lamina, photoreceptor cells degenerate about two weeks after eclosion, indicating the existence of a possible retrograde trophic factor (Campos et al., 1992). The decreased rate of survival, seen here after two weeks in vitro, of cultures prepared from early pupal eye-disc fragments, even those cultured in the MM3 medium, might result from the lack of this postulated factor from the lamina, a possibility that may now be tested using co-culture experiments.
Short-term cultures (lasting at least 5d) were also established with L-15 media, either supplemented with 10% FBS, or in a combination of 27% L-15 and 73% *Drosophila* Ringer's solution supplemented with 5% FBS. These two combinations did not allow cultures to survive satisfactorily in the longer term, however.

One particular difference between the compositions of L-15 and MM3, that may have accounted for the superior survival in MM3, is the organic composition and osmolarity of the latter medium. The L-15 medium is a vertebrate culture medium. As mentioned in the Introduction, the composition of insect haemolymph differs from vertebrate body fluids in several aspects. Insect haemolymph contains a much higher concentration of amino acids, which together account for 40% of the total osmolarity of haemolymph, compared with vertebrate fluids in which they only account for 1% (Martignoni, 1960). As a result, the total osmolarity of L-15 has a much larger osmotic contribution from NaCl (273.8 mOsm) than is true for MM3, even though the concentration of NaCl in L-15 is approximately equal to that found in larval haemolymph (summarized in Stewart et al., 1994). In L-15, the medium has a total molarity of about 177 mM, and if the inorganic salts completely dissociated in aqueous solution the total osmolarity of the medium would be about 300 mOsm. In reality this is not possible, for two reasons: a) not all the inorganic salts are completely dissociated in the medium; whereas b) some of the amino acids are in salt form, and so will contribute two ions to the total expected osmolarity. The MM3 medium (with a total molarity of about 240 mM), would be expected to contribute a total osmolarity of about 308 mOsm, if its inorganic salts were complete dissociated in solution. This is about the same as L-15, but the osmolarity of MM3 as measured by Shields and Sang (1970) is in fact hypotonic to both *Drosophila* haemolymph (Stewart et al., 1994) and to L-15 (Martignoni, 1960). Nevertheless this medium has a total amino acid concentration that more closely resembles *Drosophila* larval haemolymph (Shields and Sang, 1970), even if it has a concentration of NaCl that is much less. These differences between the MM3 and L-15 media may be the reason that the eye-
discs survive better in MM3 medium, but a major parametric study would have been necessary to demonstrate this point. The immediate empirical finding, that MM3 promotes superior survival amongst eye disc cells, was instead used to examine various aspects of cellular differentiation.

4.1.2. Neurite outgrowth from cultured eye discs

Neurite outgrowth originated from the cultures grown in the MM3 and L15 media. These neurites were immunoreactive to 24B10, which is specific to the product of the chaoptic gene in photoreceptors (Zipursky et al., 1984, 1985), and are thereby confirmed to be axons of the photoreceptors. Immunoreactivity was observed despite the envelopment of neurites by glia-like non-neuronal sheets, which the antibody must therefore have penetrated to reach the epitope on the photoreceptor membrane.

Neurite outgrowth from eye-disc fragments is greater than from entire discs in culture. Each eye-disc fragment constituted about a sixth part of the disc, but generated more fibres than from an entire disc culture (Compare Figs. 3.3 and 3.8). In fragments, therefore, a larger proportion of the clusters grew neurites that extended from the culture than was true for the intact disc. Both types of cultures required section of the optic stalk to separate the eye disc from the larval brain, however. In addition, disc fragmentation must cut many photoreceptor axons closer to their ommatidial clusters than in the intact disc. Therefore, the retrograde degeneration of photoreceptor cells resulting from section of their axons during dissection could not explain the less vigorous neurite outgrowth from intact disc cultures. Possibly epithelial cells seal the cut end of the optic stalk, mechanically obstructing neurite outgrowth onto the culture substrate from ommatidia of an intact disc.

4.1.3. Age of animals

It proved important to select animals of the appropriate age for establishing cultures. Bodenstein (1939) had already reported results from in vivo cultures indicating that the
differentiation of imaginal discs from stages younger than prepupae was very limited, while those from the prepupal stage were able to differentiate more adult characters (see also Gottschewski and Fischer, 1939). The results of eye disc pigmentation in my culture system support this view. Eye discs from P+5 or older pupae deposit ommochrome close to the time at which in vivo pigmentation would occur, while discs even from animals two hours younger, at P+3, were delayed in their pigmentation to about 10h. This temporal discrepancy increased when yet younger stages were used. In late third-instar larval organ cultures of eye-antennal discs, which were attached to the cephalic complex, ommochrome formation was delayed about four days (Schneider, 1963, 1964), compatible with my observations, even though Schneider used a different medium. This progressive increase in the delay in pigmentation in cultures taken from younger animals occurs even in the presence of 20-HE, and is possibly caused by the lack of a particular hormonal or metabolic requirement normally found in vivo and required for normal eye disc differentiation, or by the lack of regulation of such factors at critical periods of development in vitro. Cultured eye discs from pupae which have passed these periods, it is supposed, are able to differentiate more normally.

Discs from old pupae are also of limited utility. For example, neurite outgrowth is scarce from a typical culture of eye-disc fragments from P+24 (Fig. 3.11). Such eye discs will of course be further advanced in their differentiation at the time of culture initiation, but to the point that their photoreceptor axons will already have grown out from the disc (Meinertzhagen and Hanson, 1993). Neurite outgrowth in cultures established from this stage of pupal development must then result only from the regeneration of axons that had previously grown in vivo. The regenerative capacity of photoreceptor axons from animals of this age must be poor, given that the outgrowth is so sparse. It is not clear whether regeneration from all photoreceptors in old discs is inferior to regeneration from the previously grown photoreceptors of younger discs, i.e. whether the regenerative abilities of photoreceptor axons diminishes with age. This issue could be resolved by the
time-lapse observation of neurite regeneration from cell cultures dissociated from eye discs of different age. Observation of regeneration from the severed proximal neurites of photoreceptors in P+5 eye disc fragments that are partially dissociated by low concentrations of trypsin (Li et al., 1995; and see Results 3.3.1) directly confirms the ability of photoreceptors to regenerate their axons.

4.1.4. Patterning in the eye disc and movement of the morphogenetic furrow in culture

The demonstration of BrdU incorporations in cultured eye discs is critical to document the continuation of a wave of retinal development in vitro. Following a double pulse of BrdU, two separate patterns of incorporation would have been expected, both corresponding to the position of the second mitotic wave at the time of the incorporation. One would expect, therefore, a double band of incorporation posterior to the morphogenetic furrow, and the corresponding scattered incorporations anterior to this. In fact, only a single band of incorporations was seen after a double pulse of BrdU. There are two possibilities for this result. The first is that the culture conditions were not suitable for cell division, and the eye disc lost its mitotic activity during the period of culture, leaving no dividing cells to be labeled by the second pulse. The second is that the culture conditions might have been able to sustain mitotic activity in the eye disc but mitoses had ceased normally, because the second mitotic wave had reached the anterior margin of the disc, which happens in vivo 10h after pupariation (Campos-Ortega and Hofbauer, 1977). As a result, the second BrdU pulse came too late to label another band. Application of a single late pulse between 6h and 8h of culture implies the second possibility (Fig. 3.12E), suggesting that the culture conditions can support cell division at least up to 6-8 hours in vitro. The number of such cells incorporating BrdU was however reduced, as the wave approaches the anterior margin of the prospective eye field of the disc. Still, both possibilities for the single band of incorporation seen after the double BrdU pulse may be
correct, and the weakened band of incorporations both of the second pulse in the double pulse labeling experiment (Fig. 3.12D) and of the single late pulse (Fig. 3.12F), compared with the band of incorporations after a single early pulse (Fig. 3.12B), may result from a decrease and a progressive desynchronization in mitotic activity in culture. Nevertheless there can be no doubt that mitotic activity continues in culture, and such activity is further supported from cultures initiated at P+5 by the formation of inter-ommatidial bristles (see Chapter 3. Result II), the cell divisions for which commence in vivo only at P+14 (Wolff and Ready, 1993). For this reason the double-pulse cultures were not repeated.

The progress of the morphogenetic furrow, and its approach to the anterior eye margin, across the developing eye disc, is non-linear. This is because the anterior part of the disc will give rise to head epidermis, and the progenitor cells of the anterior part of the retina are in fact compressed into a relatively narrow strip that lies within and anterior to the morphogenetic furrow. Thus the furrow itself does not appear to move significantly; rather it is the posterior part of the eye field, in which ommatidial clusters have started to form, that expands, behind the furrow (Ready, 1989).

Pattern formation and the movement of the morphogenetic furrow were seen in eye discs from third-instar larvae, as shown unequivocally in Figure 14. Although in vitro differentiation of imaginal discs from this stage was limited (section 4.1.3), the patterning of ommatidial clusters continued in the cultured eye discs with a rate similar to the rate of formation in vivo. Nearly all aspects of cellular differentiation in the eye disc are initiated after pattern formation. The different influence of the same in vitro environment upon the formation of screening pigment and upon ommatidial pattern formation which was seen in cultures from third-instar larvae, suggests that cellular differentiation and pattern formation have different nutritional requirements, and that the culture conditions used are sufficient to support earlier differentiation (pattern formation), but insufficient to support some aspects
of later cellular differentiation, which therefore fail to replicate their in vivo temporal sequence.

4.1.5. The origin of floating spherules in eye disc culture

Some of the floating structures seen in eye disc and fragment cultures are cellular in nature, at least initially, but not all of them are so. They are small, and even those that are nucleated (the larger ones) must have little cytoplasm. Their appearance is similar to the structures observed by Schneider (1964), and described by her as migrating lymph gland cells in organ cultures of the eye disc and cephalic complex. The fact that they are seen in cultures of eye discs alone indicates that they do not originate from the larval brain, however, and the cell type from which they arise within the disc is not clear. Apparently, at least some must be neural in origin, since they also express a neural antigen. Cells may also migrate along the extending neurites but because floating structures also appear surrounding cultures lacking neurites, this cannot be an exclusive path. The size of most of the spherules is too small to be photoreceptor cells. A small proportion of the spherules have a diameter around 5 μm, however, and conceivably these could be migrating photoreceptor cells from the disc fragment. In a possible precedent, migrating cells are also seen surrounding cultures of other imaginal discs or their fragments (e.g. Culkin and Milner, 1991), and these have been claimed to be cells migrating from the disc epithelium. The migrating profiles are more obviously cellular in shape and comprise cell types that differ from those seen here amongst the spherules. Moreover, if the spherules are migrating photoreceptors, one would have to assume that the photoreceptors could first of all disengage from their clusters, in contrast the formation of new clusters that have been seen elsewhere, in entire-disc cultures (see Section 3.1.2D). Alternatively, there is another population of cells associated with the retina, the subretinal cells (Wolff and Ready, 1993; see Section 3.1.2C), which could presumably migrate out from the cultures, but if spherules derive from subretinal cells this would not explain why they express
photoreceptor antigens. Possibly these antigens are membrane-bound cellular debris that is slowly released from the photoreceptors, possibly as the result of phagocytic action of subretinal cells. The phagocytic action of the subretinal cells has not been previously claimed, however.

4.1.6. Cultures of optic lobe cells

The ability of optic lobe cells from third-instar larvae to survive and grow well in both the MM3 and L-15 media which have been supplemented with serum suggests that these cells are less demanding in their nutritional requirements than cells from the eye-imaginal discs. Alternatively, those cells of the optic lobe that are less discriminating in their nutritional requirements, or better matched to those available in vitro, may be those that survive in my cultures. Most of the cells seen in the culture were neurons because they are immunoreactive to anti-HRP antibody, a specific insect neuronal marker (Jan and Jan, 1982). Because the cell cultures were established from the lateral pole of late third-instar larvae, the cultures should also contain glial cells (Winberg et al., 1992). These glial cells have not been revealed immunocytochemically in my culture, because no marker for these glial cells was available. An antibody to the epithelial glial cell population (monoclonal antibody nb236) does exist, however (Hofbauer and Buchner, personal communication). Nevertheless, the number of these glial cells, visible by differential interference microscopy but immuno-negative to anti-HRP, must be low. Not only were nearly all the cells seen anti-HRP positive, but most were also monopolar in shape.

The presence of mitotic activity and neuroblast-like cells in the culture suggests that the culture conditions can support both the differentiation and proliferation of cells from the optic lobes. The decline in the number of BrdU incorporations during the culture period correlates temporally with the reduced number of neuroblasts in the outer and inner proliferation centres of the optic lobes (Meinertzhagen and Hanson, 1993). Although the possibility cannot be excluded that the culture conditions could not mai...
amongst these cells, the demonstration of mitotic activity and the advance of pattern formation seen in eye-disc cultures suggests that the decline in BrdU incorporations amongst optic lobe cell cultures most likely resulted from a decline in the number of neuroblasts, similar to the situation in vivo.

The neurons present in the cultures do not contain a homogeneous cell type. In wild-type *Drosophila*, the optic lobes contain about a hundred classes of neuron (Fischbach and Dittich, 1989). These neurons have been classified according to the positions of their cell bodies, the characteristics of their arborization and the projection area of their neurites (Fischbach and Dittich, 1989). The fact that each neuropil contains more than one type of neuron, is one of the most obvious causes for the presence of heterogeneity in the optic lobe cell cultures. The lamina and distal medulla are derived from the outer proliferation centre of larval optic lobe, while the proximal medulla and lobula complex derived from the inner proliferation centre (see Introduction). Neuroblasts from the outer proliferation centre alone would therefore give rise to both lamina (at least five monopolar cell types, and glia) and distal medulla neurons (several tens of cell types). The close relationship between the outer and inner proliferation centres in the larval optic lobe in fact makes it hard to separate the two proliferation centres during preparation of the cultures. The dissociated optic lobe cell cultures obtained could therefore contain cell progeny from both proliferation centres, further adding to the complexity of the cell types in the culture.

Within the cellular diversity of the optic lobe cell cultures, the number of cell types may be smaller than at first glance. The number of lamina monopolar neurons in the optic lobe cell cultures, for example, is probably limited. This is because the development of both lamina and distal medulla is highly dependent on photoreceptor innervation (Meyerowitz and Kankel, 1978; Fischbach, 1983) (see Chapter 1, 1.2.2.C). In particular, the production of postmitotic lamina cells depends on innervation from the retina (Selleck et al., 1992), and is therefore unlikely to occur in vitro. So, in addition to there being far fewer lamina cells than medulla cells (Hofbauer and Campos-Ortega, 1990), no new lamina
cells are expected to be born in vitro, making it rather unlikely that lamina cells occur in my cultures. Depriving the optic lobe cells in the cell cultures of photoreceptor axon innervation, should have similar effects to those of eyeless mutants of medulla cells, some of which at least differentiated normally (Fischbach, 1983). Because photoreceptor innervation is not continuously required for the survival of lamina monopolar neurons (Kunes and Steller, 1991) some lamina monopolar neurons which had already received photoreceptor innervation before the dissection procedure for culture preparation, should be able to survive in culture even without further innervation from the photoreceptor axons.

II. Effects of 20-hydroxyecdysone on eye-disc differentiation

The effects of ecdysterones on selected aspects of neuronal differentiation in vitro have been assayed for photoreceptors as well as on non-neuronal differentiation. Only in some cases were these effects sensitive to 20-HE. Moreover, no attempt was made to test the specificity of the action. This could have been done by substituting: a) other ecdysteroids in the medium, to test whether the action was specific to the 20-HE; b) other steroids, to test whether the action was ecdysteroid-receptor mediated, as opposed for example to promoting some nutritional need of the cells. Not only would this have required an impossible duplication of all culture conditions, but the literature on differentiation of insect tissues both in vivo (Riddiford, 1993) and in vitro (Milner and Sang, 1974) supports an action of 20-HE which is specific.

4.2.1. The maintenance of photoreceptor differentiation in cultures

The culture conditions used permit the differentiation of photoreceptors. An important feature of eye-disc development during metamorphosis occurs when the previously non-neuronal eye disc epithelium acquires neuronal properties. Once photoreceptor cell commitment has occurred, neuronal differentiation, as revealed by 22C10 immunoreactivity, starts at the most posterior edge of the imaginal disc at the early
third instar (Zipursky et al., 1984), and follows the posterior-anterior advance of the wave of pattern formation laid down by the morphogenetic furrow (Ready et al., 1976). Eye-discs from early pupal stages (P+3 and P+5) were used for initiating cultures in this set of studies. At these stages, the morphogenetic furrow has nearly reached the anterior edge, so that the array of ommatidia has been almost established and most neuronal differentiation in the disc has been at least initiated. Cultures of fragmented eye-discs from such stages have extensive process outgrowth in vitro which can be maintained for weeks in our culture conditions (Li and Meinertzhagen, 1995). Using anti-HRP (Jan and Jan, 1982) and 22C10 (Zipursky et al., 1984), two early onset neuronal markers, the outgrowth has been demonstrated to occur from neurons. Immunoreactivity to 24B10 further provides evidence that these outgrowing neurons are photoreceptor cells.

4.2.2. The staining patterns of 22C10 and 24B10

Even though photoreceptors in the cultures are immunoreactive to both antibodies 22C10 and 24B10, the staining patterns exhibited to the two antibodies differ in several respects. Both antibodies label membrane antigens on the photoreceptor and its extending neurites, but the neurites revealed by 22C10 are smooth and taper off towards the end of the neurite, with no immunoreactivity in the growth cone itself. By contrast, 24B10 exhibits spiny neurites with clear varicosities, growth cones and their filopodia. Potential photoreceptors acquire a neuronal identity prior to becoming photoreceptors, as revealed from the sequence of their differentiation of antigenicity to monoclonal antibodies (Zipursky et al., 1984). Eye-disc cells in vivo express antigens to 22C10 first, followed by the expression of antigens to 24B10. The pattern of expression of 24B10 antigen that is seen in vitro might seem at first sight at odds with this sequence of expression because 24B10 in vitro delineates growth cones and filopodia completely whereas the earlier expressed 22C10 antibody does not appear on the growth cone at all. The reason for this difference is simply that these two antibodies detect different antigens, the neuronal antigen
for 22C10 appearing first, but unlike the antigen for 24B10, apparently not being expressed in growth cones. This difference in the expression patterns of the antigen for 22C10 and 24B10 has not previously been reported from whole-mount studies in vivo, possibly because it is difficult to visualize growth cones in whole-mount preparations. It is of course also possible that the immunoreactivities of regenerating growth cones, which are observed in cultures, differ in some small details from the immunoreactivities of growth cones that are differentiating in vivo. In both cases immunoreactivity is specific, in the case of 24B10 to photoreceptors and in the case of 22C10 to neurons. The failure to stain the glia-like non-neuronal sheets must mean, therefore, that these antibodies can both penetrate the sheets to gain access to the underlying neuronal epitope.

4.2.3. Dependence of cellular differentiation on 20-HE

In order to assess its dependence on 20-HE, attempts were made to detect the state of cellular differentiation in cultures. This was done from the expression of imaginal antigens. In the case of photoreceptors, for example, the cells could be detected immunocytochemically, and this staining pattern then becomes the criterion for whether differentiation has occurred, at least in a qualitative sense. Immunocytochemical staining cannot readily be quantified, however, so that in cases of negative immuno-staining it was not possible to detect if differentiation were to depend on 20-HE in some subtle, quantitative way resulting in the expression of antigen only at sub-threshold levels. This is not a problem for aspects of differentiation detected by other means, such as the microscopic measurement of neurite length, but these means do not allow me the possibility to detect more interesting molecular aspects of differentiation.

Observable differences in the immunoreactivities to anti-HRP, 22C10 and 24B10 antibodies between eye-disc fragments cultured in the presence or the absence of 20-HE were not detected. This suggests that 20-HE has little effect on the differentiation of early appearing neuronal and photoreceptor epitopes in our cultures. Since neuronal
differentiation of eye imaginal discs starts in the early third-instar larva, and because the ecdysteroid titre is low during the early third instar (Riddiford, 1993), ecdysteroids appear not to be a critical factor influencing the early differentiation of neurons, even though this hormone may play an important role later on, as for example for neuronal plasticity during metamorphosis (Weeks and Levine, 1990).

4.2.1. Expression of histamine-like immunoreactivity

There is now considerable evidence for the role of histamine as a neurotransmitter at imaginal photoreceptors of Drosophila (Pollack and Hofbauer, 1991; Sarthy, 1991) and other flies (Hardie, 1987; 1989). Axons of photoreceptor cells from eye-disc fragments cultured for 6DIV express histamine-like immunoreactivity. Because the culture medium only contains histidine, the precursor of histamine, but not histamine itself, uptake of histamine from the culture medium is not possible, implying that the synthesis of histamine in photoreceptors has occurred under the conditions used in vitro. The anti-histamine immunoreactivity, however, is much weaker compared with that of adult photoreceptor axons in vivo. The weakness of the immunoreactivity makes it difficult to compare histamine expression between eye-disc fragments cultured in media with or without 20-HE supplements. It indicates, however, that differentiation of photoreceptors in this system in vitro is not completely synchronous with differentiation in vivo, or possibly that the details of subcellular storage or concentration of histamine differ under the two conditions.

It is difficult to conclude anything about the anti-histamine immunoreactivity of the cell bodies in vitro, for two reasons. The first is that the in vivo pattern of staining was not consistent. In sections of adult heads, the staining depended on which method was used. With the PAP method, axons of photoreceptors in the fenestrated layer, lamina and medulla show strong anti-histamine staining while the cell bodies of the photoreceptor do not (Pollack and Hofbauer, 1991). Using the same primary antibody with the immunofluorescence method, however, Sarthy (1991) reports intense anti-histamine
classical feature of adult synaptic ultrastructure in adult flies (Fröhlich and Meinertzhagen, 1982; and Meinertzhagen and O'Neil, 1991) was not seen in the co-culture preparation (Fig. 36). Secondly, the supposed synaptic vesicles in the co-culture preparation were much larger (70nm) than those reported *in vivo* for photoreceptor tetrads (about 30nm in diameter) (Fröhlich and Meinertzhagen, 1982; and Meinertzhagen and O'Neil, 1991). Furthermore, the putative postsynaptic neurite at the contact *in vitro* lacked the postsynaptic cisterna typical of the lamina monopolar cells L1 and L2 (Burkhardt and Braitenberg, 1976; Fröhlich, 1987). It is of course, always possible that the putative postsynaptic element from the *in vitro* preparation was not a lamina cell.
immunoreactivity in the retina as well as in the lamina and medulla. The reason for this
difference is unclear, except perhaps in small differences in fixation employed, or in
different states of light-adaptation of the flies, in the studies in the two laboratories.
The second reason is that it is not possible to attribute the fluorescence of the cultured eye-disc fragment proper to histamine-immunoreactivity of its cell bodies, which I report in
Results II (Chapter 3), because the fragments exhibit high background fluorescence. By
contrast, extending neurites exhibit clear immunoreactivity to histamine, implying that the
synthesis of histamine has commenced \textit{in vitro} in eye-disc cultures and that the synthesized
histamine is apparently transported to the tips of the growing neurites.

Anti-histamine immunoreactivity is also found in the non-neuronal envelopment
surrounding the photoreceptor axons, and this has been tentatively attributed to histamine
uptake. Glial uptake of exogenous tritiated histamine has been demonstrated in the visual
system of the locust (Elias and Evans, 1984). The uptake was in a region other than the
neuropil zones, however, which makes it difficult to predict that these glia function to take
up histamine (Elias and Evans, 1984). Nevertheless evidence from vertebrate studies
strongly suggests that glia may function to remove histamine as a neurotransmitter (Husztı
et al., 1990). Metabolic studies in the vertebrate CNS (Bischoff and Korf, 1978; Green et
al., 1987; Smits et al., 1988) suggest moreover that after being released and acting on the
postsynaptic receptor, histamine is inactivated nearly exclusively by histamine-N-
methyltransferase (HNMT). Glial cells contain abundant HNMT (Garbarg et al., 1975),
and so became good candidates to inactivate histamine after its release (Husztı et al., 1990).
Uptake of histamine has been detected in cultured chicken glial cells (Husztı et al., 1985;
1990) and in cultured rat glial cells (Rafalowska et al., 1987). Similar detailed metabolic
studies of histamine have not been carried out in \textit{Drosophila}. In locust, however,
histamine has been shown to be inactivated by N-acetylation and oxidation into acetyl
histamine and imidazole-4-acetic acid, respectively (Elias and Evans, 1983), rather than
through N-methylation, as is the case in vertebrates. It is very likely that \textit{Drosophila} uses
The appearance of synaptotagmin in axons precedes synapse formation, however (Littleton et al., 1993a). Given the apparent presence of histamine in photoreceptor neurites, the expression of synaptotagmin would at first sight suggest that the intracellular histamine may be stored in a vesicular form. The punctate pattern of synaptotagmin expression along the length of the neurite is not consistent with the continuous pattern of histamine reactivity, however. This difference suggests one of two things. Possibly, vesicles are present throughout the entire length of the neurite, but are concentrated at particular sites, and that immunoreactivity to histamine fails to demonstrate these simply because histamine immunostaining is weak. Alternatively, some histamine in the neurites may be extravesicular. It is also possible that only some vesicles express synaptotagmin.

The presence of both histamine and synaptotagmin neuronal antigens suggests the early assembly of the presynaptic machinery for synaptic transmission. Two points are noteworthy.

1) Nearly all growth cones express synaptotagmin, implying that the outgrowing neurites have presynaptic organelles at a developmental stage long before synapses would normally form in vivo. The latter occurs only after the growth cones have disappeared, during the second half of pupal development (Meinertzhagen and Hanson, 1993). Other neurons are known actually to release transmitter at early stages in their development, for example the release of acetylcholine quanta from vertebrate motor neuron growth cones (Hume et al., 1983; Young and Poo, 1983). Transmitters, possibly those released by immature neurites, can have striking effects on growth cones or on neurite growth and cell survival in surrounding neurons (Lipton and Kater, 1989).

2) The early assembly of presynaptic components in vitro occurs in the absence of the photoreceptor's normal target cells in the optic lobe. This corresponds with observations in vivo that presynaptic organelles form either in the absence of target cells, in ectopically implanted eyes (Trujillo-Cenóz and Melamed, 1975), or opposite glial cells
(Boschek, 1971). More generally, it confirms the local autonomy of the photoreceptor terminal in synaptogenesis (Meinertzhagen and Hanson, 1993).

The punctate distribution of synaptotagmin immunoreactivity along photoreceptor neurites suggests the local concentration of synaptic vesicles. This is apparently not consistent with the distribution of synaptic vesicles in the adult terminal in vivo, which occur throughout the length of the terminal (Meinertzhagen and O'Neil, 1991), but the comparison is inconclusive because there is no account of the distribution at earlier developmental stages in the pupa, with which eye-disc cultures ought more properly to be compared. Given the influence of 20-HE that we have seen on the expression of synaptic vesicle protein, the high ecdysone titre at the onset of pupariation during normal development could stimulate the formation of synaptic vesicle proteins, preparing the photoreceptor axon for either transmitter release or for synapse formation, whichever comes first.

4.2.6. Influence of 20-HE on the outgrowth of photoreceptor neurites

Several aspects of the differentiation in vitro amongst Drosophila imaginal disc cells exhibit dependence on the insect hormone 20-HE. Previous studies have concentrated in particular on imaginal disc eversion (Mandaron, 1980) and on pigmentation (Horikawa, 1958), but none has examined the sensitivity of differentiation to differing 20-HE concentrations, such as might occur in vivo during metamorphosis.

The study presented in the Results demonstrates that 20-HE influences the extent of photoreceptor neurite outgrowth in vitro. This finding confirms in a sensory neuron the demonstration in vivo and in vitro that 20-HE also regulates the arborization and neurite length of leg motoneurons, in Manduca sexta (Prugh et al., 1992; Weeks and Levine, 1990). The photoreceptors are wholly imaginal cells, however, which commence neurite outgrowth postembryonically, whereas the motoneurons are larval cells that become restructured during metamorphosis (Truman and Reiss, 1976; Levine and Truman, 1985).
It is true, moreover, that photoreceptor neurite outgrowth is mostly regenerative in my cultures, and commences only after the original axon is severed during preparation of the cultured eye disc fragment, so that in both examples 20-HE exerts an influence on neuronal regrowth, presumably as well as upon initial growth.

What is the effective concentration for the action of 20-HE? The length of *in vitro* neurite outgrowth in *Manduca* motoneurons depends on 20-HE, and in that case the lowest concentration tested to achieve a maximum effect was 1μg/ml (Prugh et al., 1992). Consistent with these findings, 20-HE also influences neurite length in the axons of cultured photoreceptors in *Drosophila*, but the maximum effect of 20-HE is attained at a much lower concentration of no greater than 0.125 μg/ml. This concentration is much lower than that of *Drosophila* prepupal haemolymph, calculated from data in Berreur et al. (1979) and summarized in Riddiford (1993) to be approximately 1μg/ml 20-HE at the prepupal stage, so that it is most likely that photoreceptor neurite outgrowth occurs under conditions of suprathreshold 20-HE concentration. Neither our results nor those of Prugh et al. (1992) have, moreover, identified the threshold 20-HE concentration for neurite growth. In both studies neurite length was measured at a single time point for all 20-HE concentrations in a particular series. Possibly, ecdysone dependence of neurite length would be revealed more sensitively during early neurite elongation, rather than for the greatest neurite extension attained, as used here.

The effects of ecdysone on other aspects of neuronal differentiation amongst the cultures studied here indicate that 20-HE can influence several aspects of the differentiation of photoreceptors prior to the formation of synapses during pupal development. These are visible at the cellular level, from the increased length of their neurites, as well as occurring at the molecular level, for example by promoting the differentiation of synaptic vesicle protein, as indicated by the increased immunoreactivity. The molecular targets of 20-HE action in the case of neurite elongation are, however, unknown.
4.2.7. Influence of 20-HE on the differentiation of non-neuronal tissues in the eye-disc culture

Pigmentation of non-visual cells in the eye disc is initiated within a narrow time window, usually about 48 hours after pupariation (Cagan and Ready, 1989). An essential role of the hormonal milieu in pigment formation was suggested long ago by transplantation experiments (Chevais, 1938) as well as in many more recent in vitro studies (e.g. Horikawa, 1958; Hanley et al., 1967; Li and Meinertzhagen, 1995). The effect of 20-HE on the differentiation of pigment cells has been suggested by various organ culture studies, in which the eye-antennal discs and cephalic complex were co-cultured together with the ring gland (Horikawa, 1958; and Schneider, 1964; Mandaron, 1970). The class of pigment granules, ommochrome or drosocptere, that differentiated in these organ culture experiments has only rarely been distinguished (e.g. Hanley et al., 1967), and insofar as ecdysone is not the only hormone secreted by the ring gland (summarized in Riddiford, 1993) the involvement of other hormones also cannot be excluded. The culture system employed here not only demonstrates that 20-HE is a particular hormone required for normal pigment formation (Li and Meinertzhagen, 1995) but exploits mutants of the pigmentary system, brown and vermilion, to examine the differential 20-HE sensitivity of ommochrome and drosocptere differentiation. In fact, the only pigment detected in the cultures was ommochrome. The drosocptere was not produced even when cultures were supplemented with the drosocptere precursor guanine, or with an extract of the adult fly. It is always possible that guanine is not the correct precursor for drosocptere formation under the culture conditions used, and that essential factors for drosocptere formation are present only prior to the adult stage and are therefore absent in the adult extract. The possibility also cannot be ruled out that the factors required for drosocptere formation were present in the adult but were destroyed during the extraction process, or that the synthesis of drosocptere is controlled by a hormone other than 20-HE. It is of interest that drosocptere formation was found in eye discs from organ cultures of the cephalic complex.
(Hanley et al., 1967), which also contained the brain hemisphere and the ring gland. I
cannot account for the difference between the results from Hanley's and my own studies.
It is possible that the presence of additional unidentified factors provided by the cephalic
complex in Hanley's study can account for drosopterine accumulation in vitro.

After 3DIV bristles formed in eye-disc fragments cultured in the presence of 1μg/ml
20-HE. Cell divisions that generate these bristles start approximately 14 hours after
pupariation in vivo (Wolff and Ready, 1993). The bristles in cultures from P+5 must
therefore have been generated from cell divisions in vitro. I was unable to detect bristles in
eye-disc cultures without supplementation with 20-HE, even though all other culture
conditions were the same. It is possible that bristles would still form in cultures lacking
20-HE, but at a much later stage of culture. Studies with ecdysoneless (Sliter, 1989;
Henrich et al., 1993) have demonstrated that ecdysteroid hormone plays an important role
in the generation and differentiation of sensory bristles across the integument during pupal
development. Although mutations in this gene usually cause pleiotropic effects (Sliter,
1989; Henrich et al., 1993), these mutational studies and the in vitro observations reported
here together suggest that ecdysone is required for normal bristle differentiation during
pupal development in vivo.

III. Regenerating photoreceptor axons in vitro and their interaction with
optic lobe cells

4.3.1. Regeneration of photoreceptor axons

The neurite outgrowth from eye-discs seen in cultures could have originated from
one of two sources, either from the regeneration of pre-existing photoreceptor axons that
had been severed during the dissection required to separate the eye-disc from the
supraesophageal hemisphere, prior to its culture, or from ommatidial clusters which are newly formed in vitro.

Evidence presented in Chapter 3 (section 3.3.1) implies that most outgrowth observed was regenerative. Observation of the attachment and elongation of neurite stumps from eye-disc fragments indeed provides direct evidence that eye-discs from P+5 are able to regenerate their axons after injuries such as dissection. On the other hand, the culture conditions used in this study are also able to support the differentiation of eye-discs from second-instar larvae (unpublished observations). This is significant because at the second-instar larval stage, ommatidial pattern formation and neurite elongation have yet to commence (Ready et al., 1976). In other words, neurite outgrowth seen in cultures from the second-instar larval stage must be the exclusive product of differentiation rather than of regeneration. Moreover, indirectly, movement of the morphogenetic furrow (Chapter 3, Result I) although limited, also suggests that neurite outgrowth must actually occur from clusters newly formed in vitro, as well as from clusters pre-existing at the time when cultures were initiated. In other words, the culture conditions employed in this study can in principle support both the differentiation and the regeneration of photoreceptor axons, with the exact blend of the two depending on the developmental stage. To my knowledge, regeneration of insect neurons in vitro has not hitherto been reported.

It is possible to calculate what this blend might be, for an entire disc on average, from the following steps: a) the developmental sequence of lamina innervation, which indicates that at P+12.5% (roughly P+12h) there are 24 rows of innervated lamina cartridges (Meinertzhagen and Hanson, 1993), leaving about 6 cartridge rows yet to be innervated; b) the assembly of new ommatidia, at a rate of one row per 1.5h (Wolff and Ready, 1993), would then mean that at P+5, the time at which most cultures were initiated in this study, there would be not 6 rows but up to 10 cartridge rows still to be innervated, but for some of these, neurite outgrowth would have already commenced from the retina; c) facet maps of Ready et al. (1976), which indicate that the 10 anterior rows contribute 204
ommatidia out of a total of 742 for the entire eye. In other words, at P+5 more than 72% (742 - 204 = 538 out of 742) of ommatidia must have commenced neurite outgrowth from the eye disc, when my cultures were initiated. This reasoning holds for the entire eye disc, but fragment cultures use only a portion of the disc. Whether the fragment cultures corresponded to posterior sectors, in which all ommatidia had already emitted axons, and from which neurite growth seen in vitro would then have been regenerative, or to anterior sectors, in which ommatidia would have differentiated their axons anew in vitro, is a matter of chance.

It is difficult to distinguish which outgrowing neurites seen in cultures of prepupal eye-disc fragment were from photoreceptors that were regenerating, and which from ones that were differentiating de novo. This is because it is not possible to follow individual axons in unstained cultures. In fragment cultures, individual fibres may be followed in some cultures occasionally, but the location of the cluster within the original eye-disc, and whether it derived from a cluster that had already formed posterior to the furrow, and was thus regenerating in culture, or whether from a cluster that commenced differentiation only after culture was initiated, is not clear. Nevertheless, the eye discs used to establish cultures were mostly from prepupae. The progression of the morphogenetic furrow is already sufficiently advanced in vivo at this stage, and most neurite outgrowth must therefore have occurred prior to initiating the cultures. In that case in vitro neurite outgrowth seen here must in most cases have been regenerative not differentiative. The rapid onset of neurite outgrowth provides additional evidence for the regeneration of photoreceptor axons. The assembly of new ommatidia in vivo takes about 1.5h per ommatidial row (Wolff and Ready, 1993), whereas neurite outgrowth is seen in vitro within 5h after initiating cultures (Results I). This period allows insufficient time for the assembly and subsequent differentiation in vitro of many new ommatidia. (With an in vivo rate of ommatidial assembly of one row per 1.5h, no more than three rows could possibly have assembled and differentiated new axons within 5h, which as calculated above
corresponds to no more than 10% of the total number of ommatidia which would be eligible to differentiate new neurites as opposed to regenerating their old ones. This calculation assumes that ommatidia assemble as rapidly in vitro as in vivo.)

Although neurite outgrowth was abundant in eye-disc cultures prepared from prepupae, it was scarce in older cultures of P+24 (Chapter II, Results I). The poor neurite outgrowth from old pupae (P+24) may imply the poor regenerative capability of old pupae. Two possible mechanisms might be responsible for a decreased capability for eye discs from older flies (P+24) to regenerate. The first is the presence of putative growth inhibitors in old pupae. It has been known for a long time, for example, that the ability of neurons to regenerate from the adult mammalian CNS is poor, while neurons from the CNS of fish and amphibians can regenerate without difficulty. Recent studies have attributed the poor regenerative capacity to the local neuronal environment (Sivron and Schwartz, 1994; Stichel and Muller, 1994). Myelin-associated growth inhibitors seen in the adult rat CNS, which is non-regenerative, have been found only at much lower levels in the optic nerve of fish (Sivron and Schwartz, 1994), which is regenerative (Sivron et al., 1994). Applying antibodies that block the activity of growth inhibitors (Sivron and Schwartz, 1994), or treatment with cytotoxic agents that eliminate oligodendrocytes, the source of production of myelin-associated growth inhibitors (Eitan et al., 1994), renders an adult rat optic nerve capable of regeneration. The regenerating photoreceptor axons seen in my cultures are wrapped by a glia-like non-neuronal sheath (Chapter 3, Results II), and it is theoretically possible that these non-neuronal structures might produce growth inhibitors. Studies in this area would however be needed to obtain further evidence of the existence of such inhibitory molecules. If inhibitory molecules do exist, the poor regenerative capacities of P+24 eye-discs could be attributed to the presence of higher levels of growth inhibitors than those in P+5. Interestingly, 20-HE enhances the outgrowth of neurites from eye discs, but inhibits the appearance of the glia-like non-neuronal sheath close to the terminal of regenerating neurites (Chapter 3, Results II). Whether increased neurite outgrowth has
any relation to the reduced extent of the non-neuronal sheath needs to be tested possibly in cultures which lack these sheathes. A second, perhaps more economical mechanism which could possibly contribute to the poor regeneration of photoreceptor axons from P+24 is the intrinsic program of photoreceptor differentiation. The regenerative capacity of a photoreceptor might merely be programmed in cells to diminish with age.

4.3.2. Autoregulation of neurite extension by histamine

The neurite length of most of the photoreceptor axons seen in my cultures is over 100μm, and some axonal fibres were longer than 200μm. These lengths are much longer than the thickness of the adult lamina which is about 30μm (Fischbach and Dittrich, 1989). Some regulatory factor must therefore be present in vivo to restrict the extent of neurite elongation and so secure an appropriate length of the photoreceptor axons, one that is explicitly conditioned in the search for connectivity mutants (Martin et al., 1995).

Interaction with target neurons is the most obvious regulatory mechanism that might stop the growth of photoreceptor axons. Neurotransmitters have also been suggested as autoregulatory molecules which influence neurite outgrowth (Budnik et al., 1989; Goldberg and Kater, 1989; Lauder, 1993). The presence of histamine at concentrations higher than 0.1mM seems to have an inhibitory effect on the average neurite length. The reduction in neurite length was mostly not statistically significant, however, so that the results presented here do not immediately confirm this view. One group did exhibit a significant reduction in neurite length, in the case of eye-disc fragments which had been cultured for 6DIV in the presence of 1mM histamine. The histamine concentration used in that group may be far above the physiological range, however, so that it is hard to reach any conclusion about the biological relevance of this result. It is entirely possible that histamine has no inhibitory feedback effect on photoreceptor axon extension, even though it may have other regulatory effects in the visual system. Confirmation of this view from
these experiments is however conditional on several uncertainties in the pharmacological action and metabolism of histamine. These are:

1) The stability of free histamine in the culture medium, and thus the stability of its concentration over the culture period. The fact that a decline in average neurite length was seen at the highest histamine concentration (1mM) after 6DIV suggests that the histamine concentration does not drop below the level required to exert a possible inhibitory action on neurite outgrowth, and that that action may have a very slow onset. The length of delay and the high concentrations required to obtain an effect imply that a possible action of histamine does not occur under physiological conditions.

In order to stabilise histamine in the medium an antioxidant such as ascorbic acid could have been used, and the rate of histamine oxidation and or degradation tracked by HPLC determinations in the medium. It is not expected that the normal route for intracellular histamine inactivation, in insects by N-acetylation and oxidation into acetyl histamine and imidazole-4-acetic acid, respectively (Elias and Evans, 1983), would occur in vitro, unless such enzymes were expressed extracellularly. On the other hand, histamine uptake mechanisms might differentiate in photoreceptor neurites, that could have diminished the extracellular concentration of transmitter.

2) If neurite outgrowth does respond to externally applied histamine, receptors for its detection would be expected on the plasmalemma, possibly of the growth cone. Such expression is known for other growth cones and transmitter systems (Davenport et al., 1993). Histamine receptors, even those postsynaptic to the photoreceptor terminal (Hardie, 1989) are not well characterised, however. Nevertheless, in common with receptors for other transmitter substances, such as the β-adrenergic receptor (Perkins et al., 1991), they would be expected to exhibit receptor desensitisation in the presence of sustained extracellular histamine, the time course for which is totally unknown in the case of the Drosophila histamine receptor.
4.3.3. Synapse formation *in vitro*

The presence of synaptic vesicle protein, synaptotagmin, and the synthesis of histamine, in photoreceptor axons regenerating from eye-disc fragment cultures both suggested that photoreceptor neurites might be able to undergo synapse formation *in vitro*. These observations are consistent with evidence from vertebrate photoreceptors. Mandell et al. (1993), for example, reported that regenerating photoreceptor axons in the salamander retina are capable of synaptic renewal. They reported that such neurites from photoreceptors in culture contained synaptic vesicles associated with microtubules. Upon K⁺-induced synaptic release, synaptic vesicle-membrane was demonstrated to be incorporated into the plasma membrane. Mandell et al. (1993) concluded that, in that case, regeneration of the photoreceptor's presynaptic terminal is independent of contact with target neurons or other cells, and that regenerated presynaptic terminals are capable of releasing neurotransmitter by exocytosis of synaptic vesicles.

Ultrastructural analysis of synaptic maturation *in vivo* indicates that synapse formation is a gradual and progressive process (Fröhlich and Meinertzhagen, 1982). Although synaptic structures are seen in the lamina neuropile as early as 74% of pupal development, many features of adult synaptic ultrastructure are missing at this stage. The T-shaped presynaptic ribbon, which is a characteristic of the mature synapse, is for example only present at 94% of pupal development.

Observation of contacts in co-cultures having an ultrastructure that resembles that found *in vivo* at identified synapses (see Chapter 3, section 3.3.3) suggested that even though the regeneration of presynaptic structure is independent of contact with other cells, it is possible that the presynaptic terminals regenerated *in vitro* are able to establish synapses with cells they contact. The absence of classical presynaptic ribbons in the co-cultures raises two possibilities: (1) the synaptic-like structure in culture might be at an immature stage, before presynaptic ribbons would normally differentiate; or (2) the ribbon might have existed at the junction seen in culture, but in sections adjacent to the one
photographed. Serial sections would have been necessary to give an answer to the second possibility, but obtaining serial sections from a culture preparation as well as searching for the area of neurite contact containing a particular synaptic site would be a more difficult task. This is because, unlike the lamina neuropile of pupal and adult flies, which is packed with highly organized neurites and their synapses, the co-cultures contain far fewer contact sites between the neurites of numerous randomly arranged cell bodies. At the moment, because of the difficulty in identifying cell profiles in EM sections of cultures it is not even known whether the synaptic contacts seen in culture are formed between a photoreceptor cell and an optic lobe monopolar cell, or whether between two photoreceptor cells. Photoreceptor axons in vitro are known to be capable of forming presynaptic ribbons upon glial cell targets (Boschek, 1971; Trujillo-Cenóz and Melamed, 1975). More important, there is no evidence of synaptic transmission in vitro. Nevertheless, the observation of synaptic organelles itself is an encouraging observation, given that to my knowledge synapse formation has not hitherto been reported in insect neuronal cultures. This observation should provide a basis for future studies of synapse formation in vitro.
Chapter 5

CONCLUSIONS

This project has established a primary culture system for the visual system of *Drosophila*. Upon screening three culture media, the best survival and neurite outgrowth has been obtained with the MM3 medium supplemented with 2% FBS. This medium has allowed the continuation of mitotic activity among the cultured cells, as well as promoting their later differentiation. To obtain ommatidial differentiation, 20-HE is required in the culture medium, and with this culture condition (2% FBS-MM3 with 20-HE), most aspects examined of the *in vivo* differentiation of the compound eye, such as pigmentation, bristle formation, and lens formation, have been replicated *in vitro*. This has enabled me to examine which cellular combinations survive and differentiate best in culture.

Different types of eye-disc culture have different degrees of utility. Eye-disc fragment cultures give rise to vigorous and consistent neurite outgrowth, and are therefore useful to study neurite outgrowth. The intact eye-disc culture retains the *in vivo* architecture of the developing eye, and is therefore useful in developmental studies involving the behaviour of the entire eye disc. With cultures of the intact eye disc, pattern formation and movement of the morphogenetic furrow have both been demonstrated to continue *in vitro*, and as a result therefore the present culture conditions can support close to normal development of the eye-imaginal disc *in vitro*. Even if the duration for which normal visual system development can be sustained *in vitro* is still not confirmed, this sets the foundation to analyse the mechanisms of development *in vivo* from findings *in vitro*.

The advantage of employing a tissue culture system is, of course, simply to be able to isolate a phenomenon of interest from the millions of other events happening at the same time and at the same location. The hormonal study presented in this thesis demonstrates that even though all the eye-imaginal disc cells are exposed to the same ecdysteroid surge during metamorphosis, not all aspects of differentiation depend on the presence of the
hormone. For example, the neuronal differentiation of photoreceptor and the extension of photoreceptor axons seem not to be regulated by 20-HE. The differentiation of non-visual pigment, lens and interommatidial bristle on the other hand do require the presence of the hormone. Other aspects of differentiation, such as the expression of a synaptic vesicle protein, synaptotagmin, require 20-HE to regulate its level, despite the fact that for expression itself to occur does not require the hormone.

The finding presented in this thesis also indicate that developing photoreceptor cells have the ability to regenerate as well as to have the potential to form synapse in vitro, capacities which would provide a valuable means to study synapse formation in vitro. Thus not only do the culture conditions support the continuation of development in cells of the visual system, but also the recovery of past developmental stages during regeneration. This demonstrates an aspect of the regenerative capacity of photoreceptor cells that has not been well documented in vivo, and thus indicates not only that developmental phenomena in vivo can be capture in vitro, but that such phenomena that can occur in vitro can also point to events occurring in vivo.

Many aspect of compound eye development in vivo can be replicated in vitro with the MM3 medium, but the culture conditions are still not optimal. For example, drosopetin does not differentiate in cultured eye discs, and the differentiation of ommochrome is delayed in cultures from young animals. Typical synaptic ribbons of adult synapses were also not seen in my cultures. These are late events in differentiation, and indicate the need for further progressive modification and improvement of the culture system.

Several immediate goals arise from the progress of this study, for which this thesis can be seen as the first stage, include the following:

1. Further improvement of the culture conditions. The beneficial effects of several components of an improved medium would include examination of a) the effects of insulin in the culture medium on cellular survival and differentiation; and 2, the use of different
substrates, such as laminin and other cellular matrix molecules on adhesion and neurite outgrowth, and the influence of these on cellular survival.

2. Study of the interactions between photoreceptors and optic lobe cells. These include: a) the possibility of synaptic interactions, or of synapse formation; b) the trophic interaction between the two cell populations, during long-term culture; c) the determination of cell fate among lamina ganglion cells, and its possible dependence upon innervation from photoreceptor axons.

3. Study of neurite fasciculation in visual systems, and the use of in vitro cultures to examine defects in fasciculation mutants.
Appendices

1. Fungicide (used in fly rearing medium)

1 ml propionic acid
1 g p-Hydroxybenzoic acid methyl ester
Dissolve in distilled water to a final volume of 500 ml.


\[
\begin{align*}
136.89 \text{ mM NaCl} \\
2.68 \text{ mM KCl} \\
0.30 \text{ mM NaH}_2\text{PO}_4\cdot\text{H}_2\text{O} \\
5.56 \text{ mM Glucose} \\
1.78 \text{ mM CaCl}_2 \\
3.94 \text{ mM MgCl}_2
\end{align*}
\]
Dissolve in 1.0 L of milli-Q H\text{\textsubscript{2}}O, and filter through 0.2\text{\textmu}m filter to sterilize.

3. Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-free *Drosophila* Ringer's (modified after Wu et al., 1983).

\[
\begin{align*}
136.89 \text{ mM NaCl} \\
2.68 \text{ mM KCl} \\
0.30 \text{ mM NaH}_2\text{PO}_4\cdot\text{H}_2\text{O} \\
5.56 \text{ mM Glucose}
\end{align*}
\]
Dissolve in 1.0 L of milli-Q H\text{\textsubscript{2}}O, and filter through 0.2\text{\textmu}m filter to sterilize.
4. Phosphate-buffered saline, PBS (0.01M)

A: Na$_2$HPO$_4$·12H$_2$O  
B: NaH$_2$PO$_4$·H$_2$O

Mix 80ml A + 20 ml B + 8.5g NaCl, dissolve in distilled water to a final volume of 1.0 L.

5. Tris-buffered saline, 10x concentration (from Benzer’s lab)

Tris  0.116 M  
NaCl  1.29 M  
KCl  0.05 M  
EGTA  0.01 M  

Dissolve in 180 ml of distilled H$_2$O. Adjust pH to 7.5 with HCl. Bring volume to 200 ml and store at 4 °C.

6. Poly/bed 810 epoxy (Epon)

Poly/bed 812 resin  25.00 g  
DDSA (Dodecenyl succinic anhydrid)  13.10 g  
NMA  13.35 g  
DMP 30 (2,4,6-tri (dimethylaminomethyl)-phenol)  1.42 g  
Mix well.
7. Lead citrate (poststaining solution)

(A) sodium citrate, Na3(C6H5O7).2H2O 0.36 M
(B) lead nitrate, Pb(NO3)2 0.24 M

3 ml (A) + 3 ml (B) + 1.6 ml NaOH (2 pellets/5 ml H2O). Shake well and make up to a final volume of 10 ml with H2O.

8. MM3 medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>34.87 mM</td>
</tr>
<tr>
<td>MgSO4.7H2O</td>
<td>16.23 mM</td>
</tr>
<tr>
<td>CaCl2.6H2O</td>
<td>6.4 mM</td>
</tr>
<tr>
<td>Na glutamate</td>
<td>46.5 mM</td>
</tr>
<tr>
<td>glucose</td>
<td>55.49 mM</td>
</tr>
<tr>
<td>Pis-Tris</td>
<td>5.02 mM</td>
</tr>
<tr>
<td>T.C. Yeastolate</td>
<td>1 g/L</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.01 g/L</td>
</tr>
<tr>
<td>Penicillin G. Na</td>
<td>0.03 g/L</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>2.25 mM</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.2 mM</td>
</tr>
<tr>
<td>Serine</td>
<td>3.33 mM</td>
</tr>
<tr>
<td>Asparagine</td>
<td>2.27 mM</td>
</tr>
<tr>
<td>Glutamine</td>
<td>4.11 mM</td>
</tr>
<tr>
<td>Proline</td>
<td>3.48 mM</td>
</tr>
<tr>
<td>Glycine</td>
<td>6.66 mM</td>
</tr>
<tr>
<td>Alanine</td>
<td>16.84 mM</td>
</tr>
<tr>
<td>Valine</td>
<td>3.42 mM</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.68 mM</td>
</tr>
<tr>
<td>Iso-leucine</td>
<td>1.91 mM</td>
</tr>
<tr>
<td>Leucine</td>
<td>3.05 mM</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.38 mM</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.51 mM</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.62 mM</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.49 mM</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.37 mM</td>
</tr>
<tr>
<td>Lysine HCl</td>
<td>4.65 mM</td>
</tr>
<tr>
<td>Cysteine HCl</td>
<td>1.65 mM</td>
</tr>
<tr>
<td>Choline Cl</td>
<td>0.36 mM</td>
</tr>
<tr>
<td>Oxaloacetic acid</td>
<td>1.89 mM</td>
</tr>
<tr>
<td>NaH2PO4.2H2O</td>
<td>5.06 mM</td>
</tr>
</tbody>
</table>
Dissolve above ingredients in 100 ml Milli-Q water and stir for 1 hr. Adjust the pH to 6.8 with NaOH. Sterilize by 0.2µm Millipore filtration. Add 2% FCS two or three days before use.


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