Structural characterisation of the *Drosophila* mushroom bodies

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Summary

The brain of the fruit fly *Drosophila melanogaster*, although only comprising some 200 thousand neurons, displays a remarkable repertoire of behavioural responses to it's environment. The relative simplicity of it's brain structure in comparison with vertebrate models or even higher invertebrates, wealth of genetic data and availability of both quantitative and qualitative behavioural assays, make it an ideal model organism for studying brain structure/function relationships. The recent development of P-element based enhancer-trap technology has provided a new tool both for visualisation and for manipulation of neurons. This technology has been used here to investigate the structure of the mushroom bodies. One of the major regions of higher function in the insect brain, the mushroom bodies have been compared to the mammalian hippocampus. Confocal microscopy of enhancer-trap expression patterns reveals neuronal structure to a higher degree of resolution than is possible using most traditional neuroanatomical techniques. A total of 31 P{GAL4} enhancer-trap lines isolated from a screen of 1800 were chosen for detailed analysis. Structural subdivisions in terms of gene expression invisible to classical neuroanatomy are evident, suggesting a possible degree of functional subdivision. The expression patterns in the larval mushroom bodies also show subdivisions. The nature of the subdivisions are different at the two developmental stages. Analysis of the developing brain during the pupal stages illustrates the structural re-organisation of the mushroom bodies during this period. Hydroxyurea ablation of the mushroom body neuroblasts in the early larvae results in a small remnant that survives from the embryo to the adult. Extrinsic output from the mb lobes and input to the calyx appears unaffected by the ablation of the mushroom bodies. Partial ablation of the neuroblasts provides evidence that the four fold symmetry of the mb structure is a reflection of the clonal nature of each of the four clusters and tracts and that the mushroom bodies are closely related to the lower order olfactory centre, the antennal lobes.

Table of Contents

Summary 2
Table of Contents 3
Figures and Illustrations 7
Abreviations 8
Acknowledgements 10
Author's Declaration 11

Chapter 1. Introduction 12
1.1 Drosophila melanogaster 12
1.2 The Drosophila Brain 13
1.2.1 Basic Organisation 13
1.2.2 Neuroanatomical approaches. 16
1.3 The Mushroom Bodies 19
1.3.1 The Structure of the Mushroom Bodies. 19
1.3.2 Development of the Mushroom Bodies 23
1.3.2.1 Origin from 4 neuroblasts 23
1.3.2.2 Reorganisation during metamorphosis 25
1.3.3 Mushroom Body Function. 28
1.3.4 Mushroom Body Structural Mutants. 31
1.4 Enhancer traps 32

Chapter 2. Materials and Methods 36
2.1 Drosophila 36
2.1.1 Fly stocks and rearing conditions 36
2.1.2 Timed pupal series 37
2.1.3 HU Ablation 38
2.2 Visualisation techniques 38
2.2.1 Immunohistochemistry 38
2.2.2 Conventional Microscopy 41
2.2.3 Confocal Microscopy 42
2.3 3D Reconstruction and Image Processing 42

Chapter 3. The Adult Mushroom Bodies 44
3.1 Introduction 44
3.2 Enhancer trap expression patterns in the mushroom bodies 44
3.2.1 GAL4 expression in Kenyon cells 44

3.2.2 GAL4 expression in single, exemplary lines 45
3.2.3 Other lines staining the mushroom bodies 53
3.2.4 Other brain structures stained 53
3.3 Discussion 57
3.3.1 Kenyon cell sub-division in terms of enhancer activity 57
3.3.2 Concentric sub-division of the lobes 57
3.3.3 Subdivisions in the pedunculus relate to those seen in the lobes 59
3.3.4 Subdivision in the calyx and cell body layer 61
3.3.5 Single cell projections 61
3.4 Summary 63

Chapter 4. Pupal Development of the Mushroom Bodies 64
4.1 Introduction 64
4.2 P{GAL4} expression in the larval mushroom bodies 65
4.2.1 The Lobes of the Larval Mushroom Bodies 65
4.2.2 Nomenclature used for description of the larval mushroom bodies 67
4.2.3 Expression patterns in the larval mb lobes in comparison with the adult patterns 69
4.3 Pupal development of the mushroom bodies revealed by P{GAL4} expression patterns 72
4.3.1 Fibre degeneration in the larval vertical and medial lobes 72
4.3.2 Formation of the adult lobes 72
4.4 Developmental staining in other brain regions 78
4.5 Discussion 78
4.5.1 Parallel subdivision in the larval mushroom bodies 78
4.5.2 Degeneration of the larval structure 79
4.5.3 Formation of the adult g lobe 80
4.5.4 Formation of the adult a and b lobes 81
4.5.5 Relationship between the proliferation quartet and the developing mb. 81
4.6 Summary 82

Chapter 5. HU Ablation of the Mushroom Bodies 83
5.1 Introduction 83
5.2 Effect of HU ablation on P{GAL4} expression in the adult mbs 84

5.2.1 Complete ablation of the mb expression patterns 84
5.2.2 Partial Ablation of mushroom body expression patterns 87
5.2.3 Ablation of the antennal lobes 90
5.2.4 Ablation of mushroom body extrinsic neurons 92
5.3 Discussion 94
5.3.1 Derivation of Kenyon cells from the proliferation quartet neuroblasts 94
5.3.2 Origin of surviving Kenyon cells 94
5.3.3 Representation of embryonic Kenyon cells in the adult mb structure 95
5.3.4 Clonal nature of the cell body clusters and structural four fold symmetry 95
5.3.5 Targeting of mb extrinsic elements 96
5.3.6 Ablation of the antennal lobes 96
5.4 Summary 97

Chapter 6. Discussion 98
6.1 Genetic dissection of mushroom body sub-structure using the enhancer-trap system 98
6.1.1 Genetic dissection of neuronal development using the enhancer-trap system 100
6.2 From structural to functional subdivision 102
6.2.1 Multiple computational channels within the mushroom bodies 102
6.2.2 The olfactory system as a single higher order computational unit 105
6.3 Further work 107
6.3.1 Analysis of anatomical mutations 107
6.3.2 HU ablation and other developmental stages 108
6.3.3 Characterisation of the antennal lobes and other brain regions 109
6.3.4 Single cell projection patterns 110
6.3.5 Functional characterisation of mb subunits 110
6.4 Conclusions 111

Bibliography 113

Appendix 1 Yang, M.-Y., Armstrong, J.D., Vilinsky, I., Strausfeld, N.J. and Kaiser, K. (1995). Subdivision of the *Drosophila* mushroom bodies by enhancer-trap expression patterns. *Neuron*, 15, pp 45-54.

Appendix 2 O'Dell, K.M.C., Armstrong, J.D., Yang, M.-Y. and Kaiser, K. (1995). Functional dissection of the *Drosophila* mushroom bodies by selective feminisation of genetically defined sub-compartments. *Neuron*, 15, pp 55-61.

Figures and Illustrations

No.		Page
1.1	Outline of the major *Drosophila* neuropil	14
1.2	Schematic view of the mushroom bodies	21
1.3	Neuroblast activity during *Drosophila* development	24
1.4	Kenyon cell body and fibre number during development	27
1.5	The P{GAL4} Enhancer-trap system	35
3.1	Confocal planes of sections	46
3.2	Line 30Y: adult brain	47
3.3	Line 201Y: adult brain	48
3.4	Line c739: adult brain	49
3.5	Line c772: adult brain	50
3.6	Line c35: adult brain	51
3.7	Line 259Y: adult brain	52
3.8	Summary outlines of P{GAL4} expression patterns in the mbs	56
3.9	Schematic respresentation of mushroom body substructure	60
3.10	3D Reconstruction of mushroom body expression patterns	62
4.1	P{GAL4} expression in the larval mbs	66
4.2	Nomenclature used for larval mbs	68
4.3	Cross sections through the pedunculus of 3rd instar larval mbs	70
4.4	Cross sections through the cell body layer of 3rd instar larval mbs	71
4.5	Line 201Y: Pupal developmental series	74
4.6	Line c739: Pupal developmental series	75
4.7	Line 30Y: Pupal developmental series	76
4.8	Line 203Y: Pupal developmental series	77
5.1	Complete HU ablation of mushroom body expression patterns	85
5.2	Kenyon cells that survive HU ablation	86
5.3	Asymetric partial HU ablation of mushroom body expression patterns	88
5.4	Symetric partial ablation of mushroom body expression pattern c739	89
5.5	HU ablation of antennal lobe line c133	91
5.6	HU ablation of γ output fibres from mb lines 43Y and 238Y	93

Abreviations

β-gal	betagalactosidase
AEL	after embryo laying
AGT	antenno-glomerular tract
AL	antennal lobe
ALH	after larval hatching
BrdU	5-bromodioxyuridine
cAMP	cyclic adenosine mono-phosphate
CNS	central nervous system
EM	electron microscopy
HU	hydroxyurea
L-NB	lateral neuroblast
LUT	look up table
PBS	phosphate buffered saline
PKA	protein kinase A
PQ-NB	proliferation quartet neuroblasts
UAS_G	upstream activation signal (GAL4)

ant lo	antennal lobe
ant mech	antenno-mechanosensory region
cal	calyx
cbs	cell body layer
co	cortex
eb	ellipsiod body
fb	fan-shaped body
i m pr	inferior medial protocerebrum
in v bo com	inferior ventral body commisure
l deu	lateral deutocerebrum
lp	lateral protocerebrum
pb	protocerebral bridge
ped	pedunculus
n	noduli
s ar	superior arch
s l pr	superior lateral protocerebrum
s m pr	superior medial protocerebrum

sp	spur
su e ct	superior ellipsoid connective
su oes b	superior oesophageal bundle
trito	tritocerebrum
v l deu	ventro-lateral deutocerebrum

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Author's Declaration

The study described here has not been presented for any other qualification or degree. All work was carried out by the author, with only the following exceptions.

The HU treatment of first instar larvae described in Chapter 5 was performed by Steven deBelle during a period of collaborative research. All subsequent staining and analysis of treated flies was performed by the author.

Appendix 1 describes a period of joint work carried out with Ming-yao Yang that overlaps somewhat with the study described in Chapter 3. The mutagenesis and subsequent screen by staining frontal cryostat head sections with X-gal was a joint effort. The immunohistochemical analysis and confocal imaging described partially in Appendix 1 and in more detail in Chapter 3 was solely carried out by the author.

Chapter 1. Introduction

1.1 *Drosophila melanogaster*

The frequent selection of the fruit fly *Drosophila melanogaster* for genetic analysis has established it as one of the major model organisms for genetic research (Lindsey, D. L. and Zimm, G. G., 1992). In the laboratory, *Drosophila* require little for survival other than some commonly available food products such as bananas. grapes and yeast (Ashburner, M., 1989). These simple culture conditions have made their study feasible in the most primitive of laboratories. They are now more commonly cultured in small plastic vials containing food mixtures based on corn meal, yeast, agar sugar and water. Their short life cycle at room temperatures facilitates quick genetic analysis. Thus the genetic basis for all aspects of *Drosophila* has become one of the most understood, particularly amongst eukaryotes.

The brain of the fruit fly *Drosophila melanogaster* although, comprised of only some 200 thousand neurons, displays a remarkable repertoire of behavioural responses to it's environment (Heisenberg, M., 1989). The relative simplicity of it's brain structure in comparison with vertebrate models or even higher invertebrates, wealth of genetic data and availability of both quantitative and qualitative behavioural assays, make it an ideal model organism for studying brain structure/function relationships. Among these are visual and olfactory associative learning (Davis, R. L., 1993), courtship rituals (Hall, J. C., 1978), and sophisticated visual/motor co-ordination (Heisenberg, M., 1989).

1.2 The *Drosophila* Brain

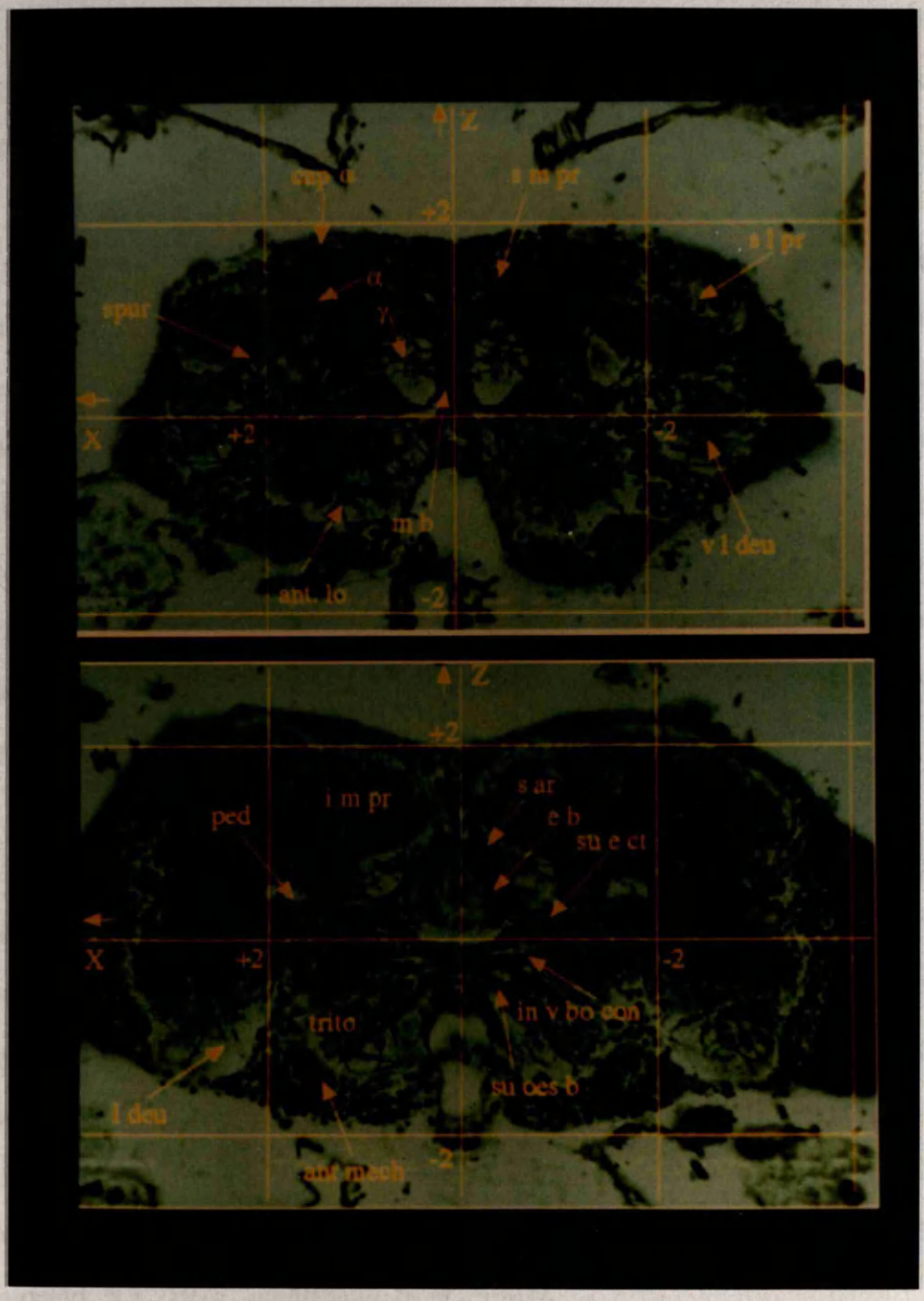
1.2.1 Basic Organisation

Despite being one of the most commonly studied organisms in biological sciences, the brain of *Drosophila* (and that of other insects), has been poorly described. There are few review texts, mainly focusing on the neuronal architecture in larger insects, most notably those for *Apis* (Mobbs, P. G., 1981) and *Musca* (Strausfeld, N. J., 1976) which contain only passing mentions of *Drosophila*. The basic structure of the *Drosophila* brain appears to share much in common with those of other arthropods (Strausfeld, N.J., 1976, Gupta, A. P., 1987). The neuronal cell bodies are in an outer layer or 'rind' called the cortex (fig. 1). Unlike mammalian neurons where both dendrites and axons extend from the neuronal cell body, in insect neurons, a single process called a neurite extends from the cell body and enters the central main brain mass. From these neurites arise dendrites and axons that collectively form tracts, and nuclei of regions of arborisation, more commonly known as neuropil (Power, M. E., 1943, Strausfeld, N.J., 1976). The difference between tracts and neuropil is poorly defined in the literature. The term neuropil has been applied to obvious structures with many tightly packed fibres that may be enclosed in glial sheaths. Tracts appear to be less tightly packed and run between the neuropil regions. Many of the arborisation patterns described in insect neurons are more extensive than their mammalian counterparts (Strausfeld, N.J., 1976). Insect neurons are mono-polar (each having only a single neurite) *in situ*, although bi-polar and multi-polar neurons have been described in *Drosophila* cell culture (Wu, C.-F., et al., 1983). Oxygen is transported to the neurons via a network of tubes called tracheoles connected to the air sacks in the head capsule. Nutrients are supplied in the haemolymph which is pumped into the head capsule from the heart.

Fig 1.1 Outline of the major *Drosophila* neuropil.

The figure shows two frontal reduced silver (Bodian) sections from N.J. Strausfeld. They have been chosen to illustrate the major neuropil regions discussed in this study. Note the layers of cell bodies surrounding the neuropil regions.

The following structures are indicated: alpha lobe (α) antennal lobe (ant lo), antenno-mechanosensory region (ant mech), tip of the alpha lobe (cap α), ellipsoid body (eb), gamma lobe (γ), inferior medial protocerebrum (i m pr), inferior ventral body commisure (in v bo com), lateral deutocerebrum (l deu), lateral protocerebrum (lp), median bundle (m b), pedunculus (ped), superior arch (s ar), superior lateral protocerebrum (s l pr), superior medial protocerebrum (s m pr), spur of the mushroom bodies (spur), superior ellipsoid connective (su e ct), superior oesophageal bundle (su oes b), tritocerebrum (trito), ventro-lateral deutocerebrum (v l deu)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The internal structures, tracts and neuropil of the brain are formed into several ganglia (Strausfeld, N.J., 1976). Three central and dorsal ganglia; the protocerebrum, deutocerebrum and tritocerebrum comprise the collective supra-oesophageal ganglia with a ventral sub-oesophageal ganglion. The optic lobes lie laterally to these structures. The ganglia within the *Drosophila* brain are fused together to such a extent, that only a rudimentary definition of each is possible (Power, M.E., 1943).

The protocerebrum is the most dorsal of the three central brain ganglia and contains structures such as the lateral horn, mushroom bodies and central complex. Of the structures in the protocerebrum, only the mushroom bodies and the central complex have been described in any detail. The mushroom bodies are postulated to be involved in olfactory associated behaviour (Erber, J., et al., 1987) and are discussed later. The central complex is the only unpaired neuropil and spans the midline of the brain (Hanesch, U., et al., 1989). It has been implicated in a variety of behaviours, in particular, visual associated memory (Heisenberg, M., 1989) and general motor activity (Bouhouche, A., et al., 1993).

The deutocerebrum is ventral to the protocerebrum and encompasses the antennal lobes. The antennal lobes are large paired glomerular centres that receive input from a number of regions, most obviously, from the antennal nerve (Stocker, R. F., 1994). They are believed to be involved in processing of chemosensory and olfactory stimuli. Fibres leave the posterior region of the antennal lobes and travel a large tract known as the antenno-glomerular tract (AGT). Fibres from the AGT terminate within the calyces of the mushroom bodies, lateral horn and lateral protocerebrum (Strausfeld, N.J., 1976, Schürmann, F. W., 1987).

The tritocerebrum lies ventral and posterior and contains the antennomechanosensory regions. This region is poorly understood and has not been described clearly in *Drosophila* (Gupta, A.P., 1987). Not all of the Central Nervous

System (CNS) structures are contained within the head capsule; within the thorax are the fused thoracic and abdominal ganglia.

1.2.2 Neuroanatomical approaches.

A large number of neuroanatomical techniques, most of which were originally applied to the vertebrate nervous system, have been applied to insects in an attempt to unravel the structure of the brain (Strausfeld, N. J., 1980). In *Drosophila*, the small size of the brain and very small cell body size of its neurons, many less than 5μm in diameter, have restricted the use of invasive techniques. Invasive techniques involve inserting a very fine glass needle into either the cell body or one of the processes. Once in place, dye can be passed through the needle into the cell to stain its processes in a procedure known as dye-filling. The needle may also be used as an electrode to electrically stimulate or record electrical activity from the neuron. However, non-invasive, histological techniques work well in the organism and have provided most of the available neuroanatomical data.

The reduced silver 'Bodian' method (Bodian, D., 1937) was used in the first comprehensive description of the *Drosophila* brain (Power, M.E., 1943) and has since been used as a standard technique for visualising brain anatomy (Ito, K., 1990). It has also been applied to the study of many other insect brains (Strausfeld, N.J., 1976). The technique provides good resolution of neuropil outlines and the tracts connecting them. However, individual fibres within neuropil regions are poorly defined and cannot be resolved. Where the fibres are narrow and tightly packaged, they stain poorly with silver. Such regions are commonly referred to as 'fine neuropil'. In some regions, the fibres are more dispersed and staining is heavier, revealing at least some of the fibres. These regions are generally known as 'course neuropil'. Although heavier staining is defined as 'course neuropil' in some

of the literature, much of this staining corresponds to regions termed 'tracts' in other texts.

Golgi impregnation , another reduced silver technique (Strausfeld, N.J., 1980), provides data on individual neuronal fibres. Entire neurons can be randomly stained during the procedure and where small numbers are stained, they can be traced through the brain and their projection patterns carefully mapped at high resolution. In such stained preparations, the terminal morphology of neurons is revealed. Close examination of the type of terminal morphology found (spined, bleb-like, etc.) has revealed differences between neurons whose overall gross morphology otherwise appear very similar (Mobbs, P.G., 1981). Golgi impregnation has provided a wealth of information on single neurons with the optic lobes (Fischbach, K.-F. and Dittrich, A. P. M., 1989), the antennal lobes (Stocker, R. F., et al., 1990), the central complex (Hanesch, U., et al., 1989) and the mushroom bodies (Yang, M.-Y., et al., 1995) of *Drosophila.*

A visualisation technique more or less specific to the adult *Dipteran* brain is autofluorescence. The red eye pigment in the wild-type fly is fluorescent. Allowing this pigment to diffuse across the brain tissue and sectioning in paraffin reveals brain structure (Heisenberg, M. and Böhl, K., 1979). Like the 'Bodian' technique, autofluorescence has poor resolution of individual neurons, although outlines of neuropil and tracts are clear. The simplicity of the technique in that it involves few steps, allows it to be performed on large numbers of flies simultaneously, in a collar, thus providing a very useful technique for mass screening.

Immunohistochemical techniques, using monoclonal antibodies against specific brain proteins, have been used to visualise brain structure(Buchner, E., et al., 1988, Bicker, G., et al., 1993). The antennal lobes comprise a number of glomerulae. These have been subdivided into functional units using this approach (Störtkuhl, K.

F., et al., 1994). A number of antibodies were raised that exhibited staining within the antennal lobes and sensilla. This provided new structural data, and a functional link between groups of sensilla and individual glomeruli. However, in general, specific immunohistochemical staining patterns using antibodies raised against known neural proteins address brain function, relying on existing structural data to provide a structure/function relationship. (Nässel, D. R., 1993, Homberg, U., 1994).

Electron microscopy has been used to provide high magnification images of many structures in the brain. Notably, it has been used to resolve much of the fine detail in the optic lobes and retinal cartridges (Meinertzhagen, I. A. and O'Neil, S. D., 1991) and the fibres running within the pedunculus and lobes of the mushroom bodies (Technau, G. M. and Heisenberg, M., 1982, Technau, G. M., 1984, Balling, A., et al., 1987).

The use of invasive techniques to dye-fill individual neurons, a technique common in larger insects, is restricted by the small cell body size and axonal diameter of most *Drosophila* neurons. However, a few larger neurons have been impaled and filled successfully (eg. Wymann, R. J., et al., 1984).

More recently, non-invasive techniques have been applied to *Drosophila* neurons *in vivo* to follow cell lineage in the developing nervous system. Of these techniques, the application of DiI to selected cells provides the most detail on individual cells (Bossing, T. and Technau, G. M., 1994). DiI, a fluorescent dye, if placed in the vicinity of a cell, will become incorporated into the cell membrane. As the cell divides, the DiI can be traced in the progeny. General staining with a modified nucleotide 5-bromodioxyuridine (BrdU) labels actively dividing cells. BrdU can be detected immunohistochemically and has been used to follow development in larger structures and tissues (Truman, J. W. and Bate, M., 1988, Prokop, A. and Technau, G. M., 1991, Ito, K. and Hotta, Y., 1992).

Radio-labelled glucose can be used for activity mapping in the brain. As active neurons use more glucose than resting neurons, by-products of radio-labelled glucose can be visualised in highly active brain regions by autoradiography (Buchner, E., et al., 1979, Buchner, E., et al., 1984, Bausenwein, B., et al., 1994). Although the resolution is low, this technique allows functional tagging of brain structures. This has been used in combination with behavioural stimuli to produce functional maps of the brain that coincide well with, and add to, the structural descriptions.

1.3 The Mushroom Bodies

This thesis mainly concerns a study of the *Drosophila* mushroom bodies (mbs). Even in 1850 when they were first described in insects, a computation role was suggested (Dujardin, F., 1850). This was based upon a resemblance between the lobed and folded mushroom bodies, and the surface of the human brain One of the most prominent features of the brain, the mbs comprise large numbers of neurons that extend processes that run in a tightly packed bundle through the brain to terminate in a series of lobes (Strausfeld, N.J., 1976). The distinctive structure, and large parallel tracts of fibres, that comprise the mbs in a range of insect species, has prompted many suggestions that they are involved in a higher brain function (for review see Erber, J., et al., 1987).

1.3.1 The Structure of the Mushroom Bodies.

Present in the adult brain of most arthropods, the mushroom bodies (also known as the corpora pedunculata or corpora fungiform) are large paired structures (one per hemisphere) located in the dorsal protocerebrum. The mushroom bodies have been described in a number of species including, *Apis* (Mobbs, P.G., 1981), *Periplaneta* (Mizunami, M., et al., 1993), *Musca* (Strausfeld, 1976) and, of course, *Drosophila*

(Power, M.E., 1943, Heisenberg, M., 1980, Yang, M.-Y., et al., 1995) The intrinsic neurons of the mushroom bodies are known as Kenyon cells.

In *Drosophila* each mushroom body in the adult brain is formed from approximately 2000-3000 Kenyon cells, the exact number being dependant on a number of factors including sex, environmental stimuli, age, density and genetic background (Technau, G.M., 1984, Balling, A., et al., 1987, Heisenberg, M., et al., 1995) . These Kenyon cell bodies, located in the posterior and dorsal region of the cortex, provide neurites that enter the calyx (so called as it is cup-shaped in other insects). The calyx receives most of its input from the antennal lobes via the anntenno-glomerular tract (AGT). The Kenyon cell axons (a single one from each neurite) emerge from the calyx to form a stalk-like pedunculus which extends to the frontal margin of the brain to bifurcate at the spur (or knee) into a dorsally projecting α lobe and a medially projecting β/γ complex lobe (fig. 1.2).

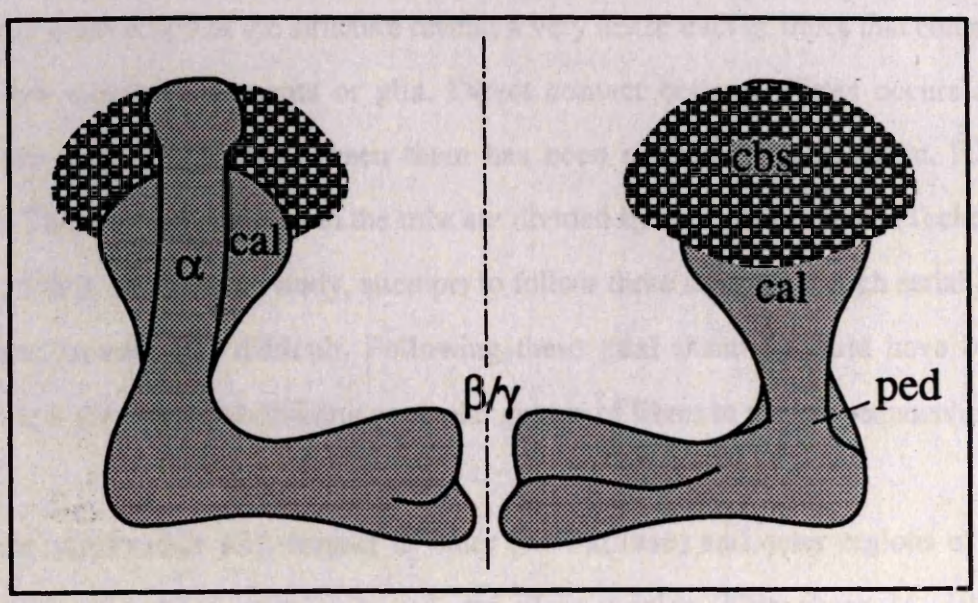


Fig 1.2 Schematic view of the mushroom bodies

The schematic view of the mushroom bodies shows the 'search' image used to select mb expression patterns (see Appendix 1). It was deduced from a reduced silver series. . Front (left) and rear (right) views of a single mushroom body. The main structural features are annotated: cell body layer (cbs), calyx (cal), pedunculus (ped), spur (sp), α lobes (α) and β/γ lobes (β/γ).

Cell body diameters of around 2-4 μm (Technau, G.M., 1984) make the Kenyon cells some of the smallest neurons described. In other insects, only a small number of glia are found in the mb cell body layer. The exact roles of these glia are unknown though they probably provide support and electrical insulation like their vertebrate counterparts (Schürmann, F.W., 1987). The small number of glia in insect mbs means that the Kenyon cells are in direct contact with each other and communication between neighbouring Kenyon cell bodies is possible. The pedunculus carries all the Kenyon cell axons yet is only around 10-12 μm in diameter (Technau, G.M., 1984). Electron microscopy of the structure reveals a very dense tract of fibres that contains very few extrinsic elements or glia. Direct contact between fibres occurs and possible communication between them has been suggested (Schürmann, F.W., 1987). The bundles of fibres in the mbs are divided by fine glial sheaths (Technau, G.M., 1984). In the same study, attempts to follow these sheaths through serial EM sections proved very difficult. Following these glial sheaths should have been possible if they were subdividing particular groups of fibres in the mb pedunculus.

Limited subdivision with respect to inner (central core) and outer regions of the pedunculus, has been revealed by reduced silver staining (Heisenberg, M., 1980). The reduced silver technique reveals how tightly packaged the neuronal fibres are. The outer regions of the pedunculus appear less tightly packed than the central ones. In bees, the mbs are subdivided longitudinally in terms of Kenyon cell arborisation pattern within the calyx, terminal morphology and transmitter expression (Schäfer, S. and Bicker, G., 1986, Schäfer, S., et al., 1988). Subdivision in terms of gene expression was revealed by immunohistochemistry against known neurotransmitters in both the pedunculus and in the lobes.

1.3.2 Development of the Mushroom Bodies

1.3.2.1 Origin from 4 neuroblasts

In *Drosophila*, the CNS develops from a group of neuroblasts that are active during specific time periods. *Drosophila* neuroblasts are large spherical cells located on the outer surface of the developing brain. Neuroblasts divide asymmetrically to produce a single, smaller ganglion mother cell with each division. The ganglion mother cell then divides symmetrically into two daughter neurons, while the remaining neuroblast continues to produce further ganglion mother cells (Bate, C. M., 1976). *Drosophila* neurons do not migrate far and are usually clustered around the region where they were formed the youngest in the centre nearest the neuroblast (Ito, K. and Hotta, Y., 1992).

Most CNS neuroblast activity is restricted to two general periods. The first in the embryo ends just prior to larval hatching, whilst the second starts late in the second instar and terminates in early/mid pupal stages (Prokop, A. and Technau, G. M., 1994). There is however, a group of five neuroblasts per hemisphere that are active from early in neurogenesis and divide continually until late in pupal development (fig 1.3). One of these neuroblasts, the 'lateral neuroblast' gives rise to neurons within the antennal lobes. The other four; the 'proliferation quartet' give rise to Kenyon cells (Ito, K., 1990, Ito, K. and Hotta, Y., 1992). Structures exhibiting all the major features of mushroom bodies are first visible in late embryonic stages and are present at all other stages of the fly's development.

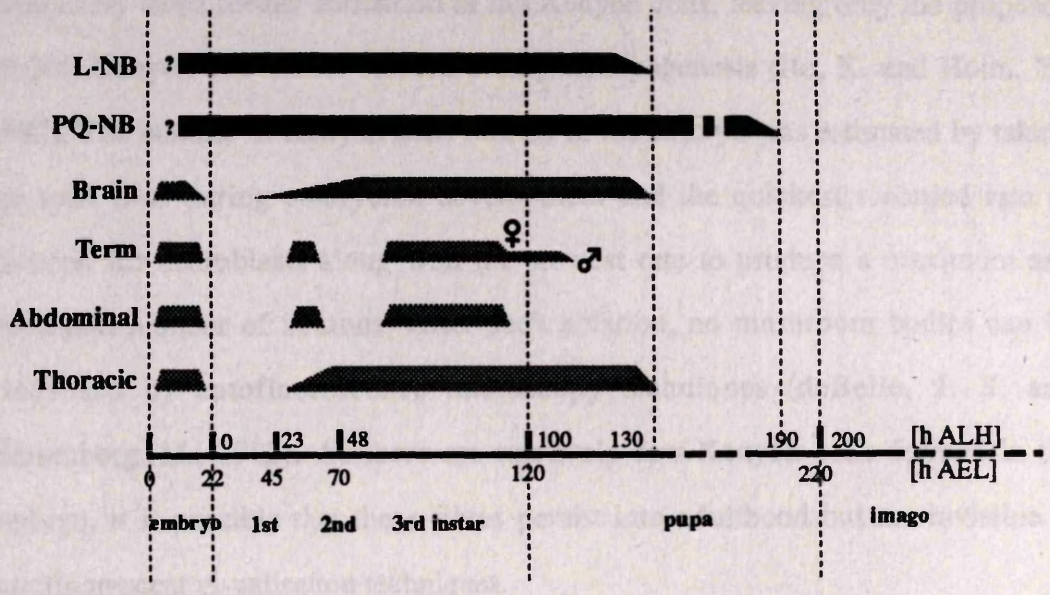


Fig 1.3 Neuroblast activity during *Drosophila* development

The figure illustrates periods of neuroblast activity (shaded bars) during *Drosophila* development. Time is indicated in both hours after larval hatching (ALH) and, hours after embryo laying (AEL). Period of activity are shown for the lateral neuroblasts (L-NB), the proliferation quartet neuroblasts (PQ-NB), general brain neuroblasts (Brain), Terminal neuroblasts (Term), Abdominal neuroblasts (Abdominal) and Thoracic neuroblasts (Thoracic).

Taken from Prokop and Technau, 1994.

Hydroxyurea (HU) kills actively dividing cells (Timson, J., 1975). As only five neuroblasts per hemisphere are active at larval hatching, a pulse of HU can be fed to the developing larvae to ablate these cells. HU ablation of the proliferation quartet completely stops further formation of mb Kenyon cells, leaving only the proposed 40-300 Kenyon cell bodies formed during embryogenesis (Ito, K. and Hotta, Y., 1992). The number of Kenyon cells formed in the embryo was estimated by taking the total time during embryonic development and the quickest recorded rate of division for neuroblasts along with the slowest rate to produce a maximum and minimum number of neurons. After such ablation, no mushroom bodies can be visualised by autofluorescence microscopy techniques (deBelle, J. S. and Heisenberg, M., 1993). As there are relatively few Kenyon cells formed in the embryo, it is possible that these fibres persist into adulthood but are invisible to autofluorescent visualisation techniques.

1.3.2.2 Reorganisation during metamorphosis

Drosophila is a holometabolous insect and undergoes metamorphosis during a pupal stage (Bainbridge, S. P. and Bownes, M., 1981). Briefly, mature third instar larvae stop feeding and seek a dry place to pupate. The larva ceases movement and secretes glue to attach itself to the side of the bottle or vial. The cuticle hardens to form the pre-pupal case. Once within this protective case, the larva moults and the proper pupal case forms within the pre-pupal (larval) one. During metamorphosis, the adult structure is formed. A large proportion of the larval structure degenerates and new structures are formed from the imaginal disks. Parts of the CNS are present in both larval and adult stages, and these can be traced through pupation (Ito, K., 1990).

During metamorphosis, the number of fibres present in the pedunculus of the mushroom bodies (counted in cross-section by EM) falls sharply (Technau, G.M. and Heisenberg, M., 1982). The reduction in fibre number was not accompanied by

reduction in the number of cell bodies, which continued to increase. This suggested a radical reorganisation of at least some of the fibres during the early stages of metamorphosis. Kenyon cell death cannot be discounted entirely, as the method used for counting the cell bodies in the study could provide no more than an estimate (fig 1.4).

Mushroom body development is dependent on a wide range of factors. *Drosophila* females have larger mushroom bodies (by volume) and also more fibres within the pedunculus (Technau, G.M., 1984). Similar differences were also observed during the same study with respect to the age of adult flies and their environmental surroundings. Fibre number was observed to increase for a week after hatching from the pupal case then decreased slowly after 3-4 weeks. Although no neuroblasts are active in the adult stage, [3H] thymidine incorporation into the mb cortex of adult flies reveals a low level of cell division, most probably of ganglion mother cells (Ito, K. and Hotta, Y., 1992). Flies kept in sensory deprived conditions, with respect to olfactory rather than visual stimuli, had fewer fibres in their mbs than those in more stimulating surroundings (Technau, G.M., 1984). A follow up study using the same fly stock revealed that other environmental conditions could induce larger mushroom bodies at eclosion. In these flies, the fibre number did not increase during the first week, but decreased after the first few days (Balling, A., et al., 1987).

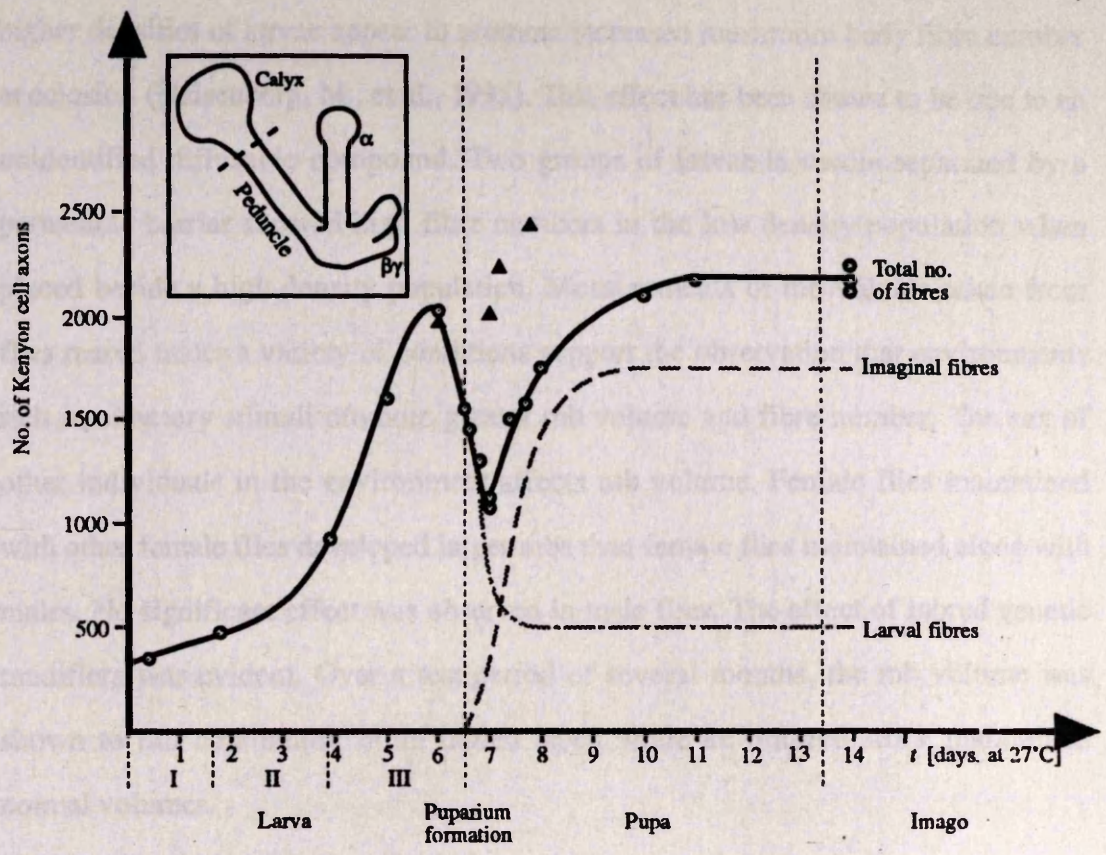


Fig 1.4 Kenyon cell body and fibre number during development

Total numbers (counted by electron microscopy) of Kenyon cell axons (including extrinsic elements) in the caudal pedunculus of the wild-type *Drosophila* (see inset for plane of section)as a function of developmental time starting at larval hatching. Axonal fibres are shown as open circles and the cell bodies as triangles. Whereas the fibres of the larval Kenyon cells degenerate within the first hours of pupal life, their cell bodies remain alive. The dash and dash-dot lines represent the hypothetical larval and imaginal fibres portions in the pedunculus. The curves are based on the assumption that (~500) thin fibres positioned centrally in the stalk system contains the only larval fibres which remain throughout metamorphosis.

Taken from Technau and Heisenberg 1984

Larval density has a profound effect on the fly. High population densities result in low body weights and longer developmental times (Ashburner, M., 1989). However, higher densities of larvae appear to promote increased mushroom body fibre number at eclosion (Heisenberg, M., et al., 1995). This effect has been shown to be due to an unidentified diffusable compound. Two groups of larvae in media separated by a permeable barrier showed high fibre numbers in the low density population when placed beside a high density population. Measurements of mb volume taken from flies reared under a variety of conditions support the observation that environments rich in olfactory stimuli promote greater mb volume and fibre number. The sex of other individuals in the environment affects mb volume. Female flies maintained with other female flies developed larger mbs than female flies maintained along with males. No significant effect was observed in male flies. The effect of inbred genetic modifiers was evident. Over a test period of several months, the mb volume was shown to fall continually in an inbred stock while an outbred stock maintained normal volumes.

1.3.3 Mushroom Body Function.

From the earliest description, the mushroom bodes have been implicated in associative learning and memory, and in controlling a variety of complex behavioural repertoires (Dujardin, F., 1850). The first direct evidence for a behavioural role for the mbs came from direct electrical stimulation, which was observed to stimulate complex and extended courtship rituals in crickets (Huber, F., 1960, Wadepuhl, M., 1983). Many studies involving either direct electrical stimulation or surgical lesioning have implicated a role for the mbs in many insect species, with a broad range of behavioural effects observed. Common features in most of these studies are disruption of elements of courtship patterns, olfactory associated behaviours and locomotor effects (Erber, J., et al., 1987).

In bees, where mb morphology obviously differs between sexes and castes, (in particular, nurse and forager castes) studies of gynandromorphs (mosaics of male and female tissue) have revealed that these structural differences are required for expression of caste specific behaviour patterns (Withers, G. S., et al., 1993). Changes in calyx size can also be observed after the first flight of honey bees (Coss, R. G. and Brandon, J. G., 1982). The α-lobe of the bee mushroom body in particular is involved in olfactory associative learning. Efferent activity from the lobe can be modified by olfactory conditioning (Mauelshagen, J., 1993). Localised cooling of the bee mbs blocks olfactory short term memory after application of the conditioning stimulus (Erber, J., et al., 1980).

In *Drosophila*, a number of single gene mutants with olfactory learning defects have been isolated. Two of these genes; *dunce* and *rutabaga* have been cloned. They are involved in the cAMP second messenger pathways (Nighorn, A., et al., 1991, Han, P.-L., et al., 1992) which, in other organisms, have been implicated in learning and memory (Hawkins, R. D., et al., 1993). This suggests a common mechanism for learning and memory between different organisms. Both genes were shown to be expressed preferentially, if not exclusively, in the mushroom bodies.

The *Drosophila DC0* gene encodes the catalytic subunit of protein kinase A (PKA), a major mediator of cAMP action. *DC0* is preferentially expressed in the mushroom bodies (Skoulakis, E. M. C., et al., 1993). Heteroallelic flies for two *DC0* mutations result in decreased olfactory learning and memory capabilities. At low cellular cAMP concentrations, PKA is composed of a tetramer of two catalytic (*DCO*) and two regulatory subunits. The catalytic subunits are released from the tetramer by binding of cAMP to the regulatory subunits, thus allowing protein phosphorylation. Over expression of the regulatory subunit inhibits PKA activity (Drain, P., et al., 1991). Such inhibition of PKA has been shown to suppress olfactory learning and memory capabilities.

As mentioned previously, the mushroom bodies can be chemically ablated by feeding newly hatched larvae hydroxyurea (HU). In such individuals, the mbs are effectively absent with the possible exception of a few fibres surviving from the embryo. HU ablation effectively abolishes olfactory associative learning in flies. Visual learning is unimpaired (deBelle, J.S. and Heisenberg, M., 1993). This treatment also kills the lateral neuroblasts which supply the antennal lobes (also part of the olfactory pathways). HU treated flies are unable to recognise at least one of the usual test odours used in olfactory associative learning tests and another odour had to be substituted. HU treatment obviously impairs antennal lobe function with respect to some odour recognition. It cannot be discounted that the effect of HU ablation on the behaviour of the flies is as a result of antennal lobe ablation rather than mushroom body ablation.

A role for the mushroom bodies in the courtship behaviour of the *Drosophila*, which relies heavily on olfaction, has been suggested. In *Drosophila* the sex of a specific cell is determined by genetic factors within the cell alone rather than from external signals. Studies of gynandromorphs, mosaics of male and female tissue, implicated the mushroom bodies, or adjacent neuropils, in control of the male courtship repertoire (Hall, J.C., 1978). The *transformer* (*tra*) gene of Drosophila has two sex specific transcripts (Boggs, R. T., et al., 1987). Expression of the female specific transcript in cells is sufficient to sexually transform those cells (McKeown, M., et al., 1988). Targeted feminisation of the mushroom bodies, by GAL4 mediated expression of *tra*, in adult *Drosophila* male flies results in them courting male flies as well as females (see Appendix 2).

The picture that emerges is of a specialised neuropil responsible for associating, storing, and retrieving multimodal sensory information, thereby providing the organism with learning abilities, memory, and predictive behaviour.

1.3.4 Mushroom Body Structural Mutants.

A number of *Drosophila* mb structural mutations have been generated through EMS mutagenesis and isolated by subsequent mass screening of lines by autofluorescent microscopy (Heisenberg, M. and Böhl, K., 1979). Although four classes of gross mushroom body structural mutation have been described, the first two overlap considerably and little, if any, work has been published on the later two classes. The mutations that have been described in detail have Kenyon cell fibres missing from the pedunculus and lobes. These fibres are usually miss-routed and tangled around the calyx. In the third class, the lobes of the mbs are fused together at the midline. In the fourth, (only one such mutant has been described) vacuoles are present along the length of the pedunculus. (Heisenberg, M., et al., 1985) Only the following few mutations have been studied in any detail.

mushroom bodies deranged (mbd)

In the adult, these flies have only a very small number of fibres (~250) in the pedunculus. The remainder appear to wrap around the calyx, forming large whirls of fibres (Heisenberg, M., 1980). At the third instar stage, the mbs appear indistinguishable from the wild-type by conventional histology. Electron microscopical analysis revealed the pedunculus to be missing a small bundle of some 400 Kenyon fibres (Technau, G.M. and Heisenberg, M., 1982). It was proposed that the bundle of fibres missing in *mbd* normally acts as a guide during pupal Kenyon cell reorganisation, and without these guide fibres, the adult fibres get miss-routed. *mbd* flies showed poor olfactory associated learning but performed well in non-olfactory associative tests.

mushroom body miniature (mbm)

In *mbm* ,the lobes and pedunculus are not visible in the adult. The calyx is much smaller than that of *mbd* and the large whirls of fibres observed in *mbd* are not visible (Heisenberg, M., 1980) As with *mbd,* the mbs appear normal in the 3rd instar stage. The mutation appears to affect the pupal development of the mushroom bodies. *mbm* is also sex-specific, the structural phenotype only visible in females. *mbm* like the *mbd* shows defects in olfactory learning tests. Interestingly, the learning defect appears in both males and females, whereas the structural defect is sex-specific (Steve deBelle *pers comm*).

mushroom body defect (mud)

In *mud* mutants several neuropil regions are affected. The antennal lobe (and AGT) is enlarged, the central complex is malformed, and the mbs have many more cell bodies. There is an enlarged calyx with Kenyon fibres whirled around it, and no visible pedunculus or lobe system (Heisenberg, M., 1980). In the larvae, there are again more cell bodies. These send fibres to the pedunculus and lobes, which appear enlarged. In *mud* mutants, regulation of neuroblast proliferation is disrupted, resulting in an increased number of mb (and other) neuroblasts (Prokop, A. and Technau, G.M., 1994). The extra number Kenyon cells from these extra neuroblasts presumably interfere with the pupal development of the mbs.

1.4 Enhancer traps

The P-element transposon of Drosophila has provided us with an ideal mobilisable vector for the introduction of DNA-constructs, encoding a wide variety of genes under various control elements into the germ-line (Finnegan, D. J., 1992, Sentry, J. W. and Kaiser, K., 1992). Wild-type P-elements are 2.9kb in length and include the gene for the transposase enzyme that facilitates transposition within the genome. This gene may be replaced with DNA encoding other genes of choice, and the

transposase provided by other means. To facilitate mobilisation of such engineered P-elements, *Drosophila* lines, such as Δ2-3 have been developed. These contain modified P-elements which express transposase at high levels, yet are unable to transpose themselves. The Δ2-3 stain used here has undergone a lesion that prevents its transposition yet it transcribes transposase at high levels (Robertson, H. M., et al., 1988).

This modified P-element technology enabled the development of enhancer-trap elements. An enhancer-trap contains a minimal promoter region, insufficient in itself, to induce transcription. If, after transposition, it inserts in a region under the influence of a local genomic enhancer, the inserted sequence (or reporter as it is known) should be transcribed in a pattern reflecting the enhancer activity. Initial enhancer-traps, P{*lacZ*} used the *E.coli* gene *lacZ* which encodes β-galactosidase (β-gal), enhancer activity then visualised simply by staining for β-gal either through immunohistochemical techniques or more simply by using the chromogenic substrate for β-gal, X-gal (O'Kane, C. J. and Gehring, W. J., 1987).

These 'first' generation enhancer-traps were of limited use in the study of neurons, due to the nuclear localisation signal attached to the reporter. The 'second' generation P{GAL4} enhancer trap works on a slightly more indirect principle (Brand, A. and Perrimon, N., 1993). Rather than visualise the reporter directly, GAL4, a transcription factor from yeast that is functional in *Drosophila* (Fischer, J. A., et al., 1988) is expressed. GAL4 can be used to drive expression of any gene that is placed downstream of the activation signal, UAS_G. A construct with *lacZ* as a reporter (without the nuclear localisation signal) was developed (fig 1.5) (Brand, A. and Perrimon, N., 1993). This allowed visualisation of cell bodies and, in neurons, axons and dendrites. In addition to allowing cell body visualisation, any DNA construct may be placed downstream of the GAL4 recognition site, thus the enhancer trap may

be used to drive expression of cellular modifiers (Moffat, K. G., et al., 1992, Ferveur, J.-F., et al., 1995).

Aims and Objectives

The overall aim of this study was firstly to further understanding of *Drosophila* brain anatomy, in particular the anatomy and development of the mushroom bodies. Secondly the study was also undertaken to isolate and characterise a number of cell specific markers for other uses such as targeted gene expression

The specific objectives of this study were to isolate enhancer-trap expression patterns that stained the mushroom bodies. Selected staining patterns were to be analysed at high resolution using an immunofluorescent approach and confocal microscopy in an attempt to reveal the structure of the mushroom body in adult *Drosophila* brain. The enhancer-trap expression patterns were then to be used as cell-specific markers to analyse the pupal development of the mushroom bodies. They were also be used in an attempt to locate any Kenyon cells that originate prior to larval hatching by treating newly hatched larvae with hydroxyurea.

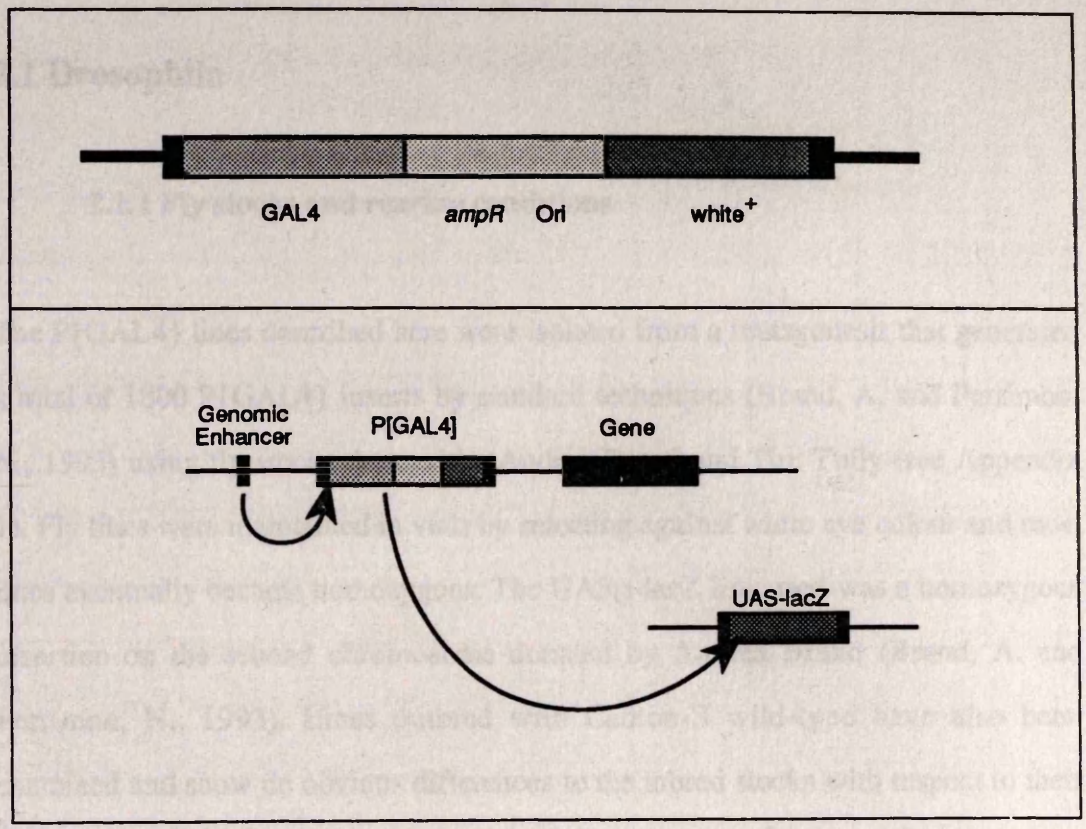


Fig 1.5 The P{GAL4} Enhancer-trap system.

(upper) The P{GAL4} element (Brand, A. and Perrimon, N., 1993)has, at either end, the short terminal regions of the wild type P-element (black regions) that facilitate transposition. Encompassed by the P-element sequences is the yeast GAL4 gene placed under the control of the minimal P-element promoter. The construct also encodes *E.coli* plasmid sequences for ampicillin resistance, an origin of replication and also an allele of the *white* gene used as a marker (red eye colour).

(lower) When inserted into the vicinity of an active *Drosophila* genomic enhancer element, GAL4 transcription is activated. GAL4 in turn can be used to activate transcription of any construct placed downstream of the GAL4 recognition sequence, UAS_G.

Chapter 2. Materials and Methods

2.1 Drosophila

2.1.1 Fly stocks and rearing conditions

The P{GAL4} lines described here were isolated from a mutagenesis that generated a total of 1800 P{GAL4} inserts by standard techniques (Brand, A. and Perrimon, N., 1993) using fly stocks donated by Andrea Brand and Tim Tully (see Appendix 1). Fly lines were maintained in vials by selecting against white eye colour and most lines eventually became homozygous. The UAS_G-lacZ line used was a homozygous insertion on the second chromosome donated by Andrea Brand (Brand, A. and Perrimon, N., 1993). Lines outbred with Canton-S wild-type have also been examined and show no obvious differences to the inbred stocks with respect to their expression patterns (Appendix 2). P{GAL4} lines were initially screened for expression in the adult mushroom bodies by taking frontal cryostat head sections of P{GAL4} X UAS_G-lacZ F1 progeny. Sections were then stained with X-gal, the chromogenic substrate for β-gal (see Appendix 1).

Drosophila were maintained in standard conditions(Ashburner, M., 1989), on a 12h dark : 12h light cycle on standard cornmeal/yeast/agar medium at 23°C-25°C and 35%-45% relative humidity. Fly density was not strictly controlled but flies were not over-crowded to the extent of visibly reducing adult body size. Fly crosses typically were of two males and three females per vial. Thus larval density was always substantially greater than five larvae per ml of food therefore the larval population density effect mb size was not relevant (Heisenberg, M., et al., 1995). When large numbers of flies were required (developmental screening) crosses were performed in bottles. Ten males were selected and crossed with 25-35 virgin

females. Parent flies were removed prior to hatching of the next generation. Unless otherwise stated, the age of adult flies examined was 4-14 days.

2.1.2 Timed pupal series

For timed pupal series, crosses were set up in bottles as above. Wandering 3rd instar larvae made their way up the side of the bottle where they would eventually evert their spiracles and stop moving (Bainbridge, S.P. and Bownes, M., 1981). These newly immobile pre-pupae were collected every 30 mins throughout the day. They were then placed on a piece of 'Scotch' invisible tape that itself was fixed to a glass microscope slide using double sided sticky tape. The 'Scotch' tape had no obvious deleterious effect on the pupae which could be lifted off the sticky tape with ease. The sex of the pupae was recorded (as judged by the size of the gonads (Ashburner, M., 1989)). The glass slide was then placed in an empty food bottle to maintain a similar temperature and humidity to that of the original bottle. The bottle was stored beside the original bottle containing the developing pupae thus under the same conditions at all times.

Male and female pupae were dissected at two and four hour intervals during pupation. The dissection times were accurate to within +/- 5 minutes, the collection times were accurate to within +/- 30 mins giving an overall accuracy of +/- 35 mins well within the +/- 1 hour used in previous studies (Ito, K., 1990). Two hourly intervals proved excessive as the changes observed were gradual and the interval was lengthened to 4 hours for most collections. The time of day for collection with respect to the constant 12hr light: 12hr dark cycle was not accounted for. Collection times were random and took place at all hours during the day and night (most commonly during the day).

2.1.3 HU Ablation

All hydroxyurea (HU) ablations were performed by Steve deBelle according to deBelle and Heisenberg, (1994). Newly emerged larvae were selected and were fed a 1 hour pulse of normal food containing HU at a concentration of either of 10mM or 50mM. After this they were transferred to a vial containing normal food with no HU. The lower 10mM concentration was used in an attempt to induce partial ablated brains where at least some of the mb neuroblasts would survive. Flies were examined as either 3rd instar larvae or as mature adults (usually at 3-5 days old).

2.2 Visualisation techniques

2.2.1 Immunohistochemistry

Intact adult brains were dissected under PBS, fixed in 4% paraformaldehyde for 30 min, and washed twice for 1 hour in PAT (PBS containing 1% Sigma cold fraction V bovine serum albumin and 1% Triton X-100). They were incubated overnight in 3% normal goat serum (SAPU) containing rabbit polyclonal anti-β-gal antibody (Cappel) diluted 1:2000 in PAT; washed three times in PAT for 1 hour; incubated overnight with secondary antibody (fluorescein-labelled goat anti-rabbit IgG; Vector Labs) diluted 1:250 in PAT; washed twice for 1 hour in PAT, and once for 5 minutes in PBS. All of the above was carried out at room temperature. Stained brains were mounted in VectaShield (Vector).

Several other variations on the above protocol were employed although none of the patterns obtained by these variations are included here.

2.2.1.1 Dissection techniques

Flies were immersed in 100% ethanol for 1 min then for a further 1 min in 1 X PBS. They were then pined to a silicon gel plate through the abdomen. The plate was then flooded with PBS and the brain dissected out of the fly by firstly removing the proboscis then teasing the head capsule apart at the remains of the mouth parts. The ethanol step facilitated this by reducing air bubbles within the head capsule and killing the fly. However, this ethanol step washed considerable amounts of the red (and fluorescent) eye pigment across the surface of the brain at this stage. This induces autofluorescence that is so strong that it interferes with the signal from fluorescein based labels. Flies that were selected for fluorescent staining were not treated with ethanol prior to dissection.

3rd instar larvae were dissected by placing the larvae in a drop of PBS on a glass microscope slide. Forceps were then used to grab the mouth parts and the mid-part of the body. The forceps were then pulled apart and the brain lifted out of the remains.

2.2.1.2 Fixation methods

Fixation can also be achieved using glutaraldehyde or paraformaldehyde. The use of glutaraldehyde as a fixative is not suitable for fluorescence as it increases the amount of background autofluorescence. Glutaraldehyde fixation was used successfully for non-fluorescent labels. Fixing using a mixture of 0.025% glutaraldehyde in 4% paraformaldehyde was found to be suitable for fluorescence though not used in this study.

2.2.1.3 Primary Antibody

Several primary antibodies were used at several concentrations. The most successful was the Cappel (USA) rabbit polyclonal antibody. This was used successfully at 1:4000 dilution in PAT though 1:2000 was used as normally less than 2mls of solution were required per day (i.e. 1 µl in 2 mls). Mouse monoclonal antibodies from Promega and Sigma were both used with poor results (using a wide variety of secondaries). Both antibodies worked at 1:1000 dilution though better at dilutions of around 1:100.

2.2.1.4 Secondary Antibody visualisation

For the Cappel polyclonal, three good secondary antibodies were used. The most commonly used one here was a fluorescein labelled goat anti-rabbit IgG antibody from Vector labs at 1:250 dilution in PAT. A Texas-Red labelled sheep anti-rabbit IgG antibody from Molecular Probes was also very successful at 1:500 dilution. A biotinylated antibody from Vector was also used. This was visualised using a VectaStain ABC Elite kit. It uses peroxidase conjugated to avidin which will bind to the biotinylated antibody. The peroxidase is then visualised by adding DAB (diaminobenzimidine). The biotinylated antibody showed some non-specific staining of brain structure. This was reduced by pre-absorbing the antibody to wild-type *Drosophila* tissue. Briefly, this was achieved by taking approximately 100 adult flies, grinding them and fixing in paraformaldhyde for 15 mins. The ground flies were then washed in PAT several times over a 2 hour period. The secondary antibody was then diluted in PAT at 1:500 and incubated with the ground flies overnight. The antibody solution was then drawn off and placed in with the brains the following day. This cleaned the staining pattern significantly. The pre-absorption step had little effect on either of the fluorescent based secondary antibodies.

For mouse primary antibodies, a mouse fluorescent staining kit containing three labelled secondary antibodies (AMCA, FITC and Texas Red) was used. Each antibody worked well at a dilution of 1:100.

2.2.1.5 Preparation Mounting

Two mountants were used; Vectashield (Vector), a liquid mountant and Glycerol-Gelatin (Sigma), a solid mountant (solid at room temp, liquid at temperatures over 40°C). For fluorescent preparations, Vectashield proved most successful as the fluorescent signal bleached rapidly in the Glycerol-Gelatin. For peroxidase/DAB stained preparations, Glycerol-Gelatin was used.

The brains were mounted between sealed coverslips. A drop of mountant was placed on the centre of a glass microscope slide. The brains were placed in this drop. A small square (22mm square, thickness 1.0 BDH) coverslip was stuck to either end of the slide using Glycerol-Gelatin. A long coverslip (22 X 64 mm thickness 1.0 BDH) was then placed on top and stuck to either of the smaller cover slips again using glycerol gelatin. The coverslip was then sealed along either edge using Glycerol-Gelatin.

2.2.2 Conventional Microscopy

Cryostat sections (Appendix 1) were examined using a Leitz Orthoplan microscope using DIC (differential interference contrast). This allowed visualisation of the outline of the entire mb structure. No obvious structural abnormalities were observed in any of the lines examined by this technique.

2.2.3 Confocal Microscopy

Whole-mount stained brains were examined with a Molecular Dynamics Multiprobe laser scanning confocal microscope. The excitation (480nm) and emission (530±15nm) barrier filters used were appropriate to the fluorescein-based label of the secondary antibody. Unless stated otherwise, either a single brain hemisphere or central brain region alone was scanned.

2.2.3.1 Autofluorescence

Autofluorescence, a technique discussed above , allows visualisation of brain neuropil by allowing the fluorescent eye pigments to diffuse across the tissue. This also happens to a limited degree if retinal tissue is not entirely removed prior to the immunohistochemical staining procedure outlined above. This allows the outline of brain neuropil to be visualised by the confocal microscope using a high laser power to reveal the autofluorescence. None of the lines examined showed any evidence of abnormally shaped or sized mushroom bodies. In addition, several lines were examined using the classical autofluorescence approach by Steve deBelle (deBelle, J.S. and Heisenberg, M., 1993). Again, the mb anatomy of these flies appeared normal (Steve de Belle, pers comm).

2.3 3D Reconstruction and Image Processing

Three dimensional reconstructions were performed using the programme 'ImageSpace 3.1' (Molecular Dynamics). Pseudo colour was added to the reconstructed view using the programme 'NIH-Image' (National Institutes of Health, Washington).

The NIH-Image pseudocolour look up table (LUT) used for the photographs in this thesis was 'Fire 1'. LUTs replace the greyscale values produced by the confocal microscope with colours. This particular LUT is a graduated colour table where low intensities are shaded black through reds, higher intensities are orange to yellow to white at maximum intensity. Thus strongly stained areas will be coloured yellow or white. Background levels of fluorescence will be black or dark red.

Montages were assembled in 'Adobe Photoshop' 2.0, 2.5 and 3.0 and also in 'NIH-Image'. All photographs were generated using a Polaroid Digital Palette Side Maker 4000 at the highest resolution setting on 100 ASA Kodak Gold Ultra film.

Chapter 3. The Adult Mushroom Bodies

3.1 Introduction

During a screen of some 1400 P{GAL4} lines, using X-gal as a stain for GAL4-mediated *lacZ* expression in frontal cryostat sections of the adult heads, 31 lines were found to exhibit preferential *lacZ* expression in the mushroom bodies. It was immediately apparent that many of the patterns of expression were different, suggesting a degree of structural subdivision (Appendix 1). As X-gal staining of cryostat sections reveals only low structural resolution, the brains were stained as whole mounts, using fluorescent immunohistochemistry and visualised by confocal microscopy. Such an approach provides both high resolution sections and 3D spatial information that can be used to construct 3D views from a variety of perspectives. The nature of these differences in staining pattern are described in detail here. They suggest a degree of structural subdivision invisible to the techniques of traditional neuroanatomy

3.2 Enhancer trap expression patterns in the mushroom bodies

3.2.1 GAL4 expression in Kenyon cells

Thirty-one lines exhibiting staining which could be followed from the cell body layer, through the calyx and pedunculus to the lobes, were stained. Such staining was judged to be of the mb intrinsic neurons, Kenyon Cells. All of the lines stained other neurons elsewhere in the brain. Some of the lines examined also stained mb extrinsic neurons. Lines 43Y and 238Y, exhibited staining of Kenyon cells and mushroom body extrinsic neurons. Both lines stained a small cluster of cell bodies located at the frontal dorsal margin of the brain, from which processes arise to

terminate within the γ lobe, and within the lateral protocerebrum. In line 43Y this appears to contribute all the γ staining. In line 238Y, the γ lobe shows staining of both intrinsic and extrinsic components (See Chapter 5 for further details).

3.2.2 GAL4 expression in single, exemplary lines

Figures 3.2-3.6 feature the most revealing of the mushroom body staining patterns. The subdivisions discussed later are all exemplified by these expression patterns. When mounting whole mount brains for confocal microscopy, the brains usually fall in such a position that the scanned sections are of an approximately frontal aspect. The approximate plane of section for each confocal image is illustrated in a saggital schematic diagram of a brain (fig 3.1).

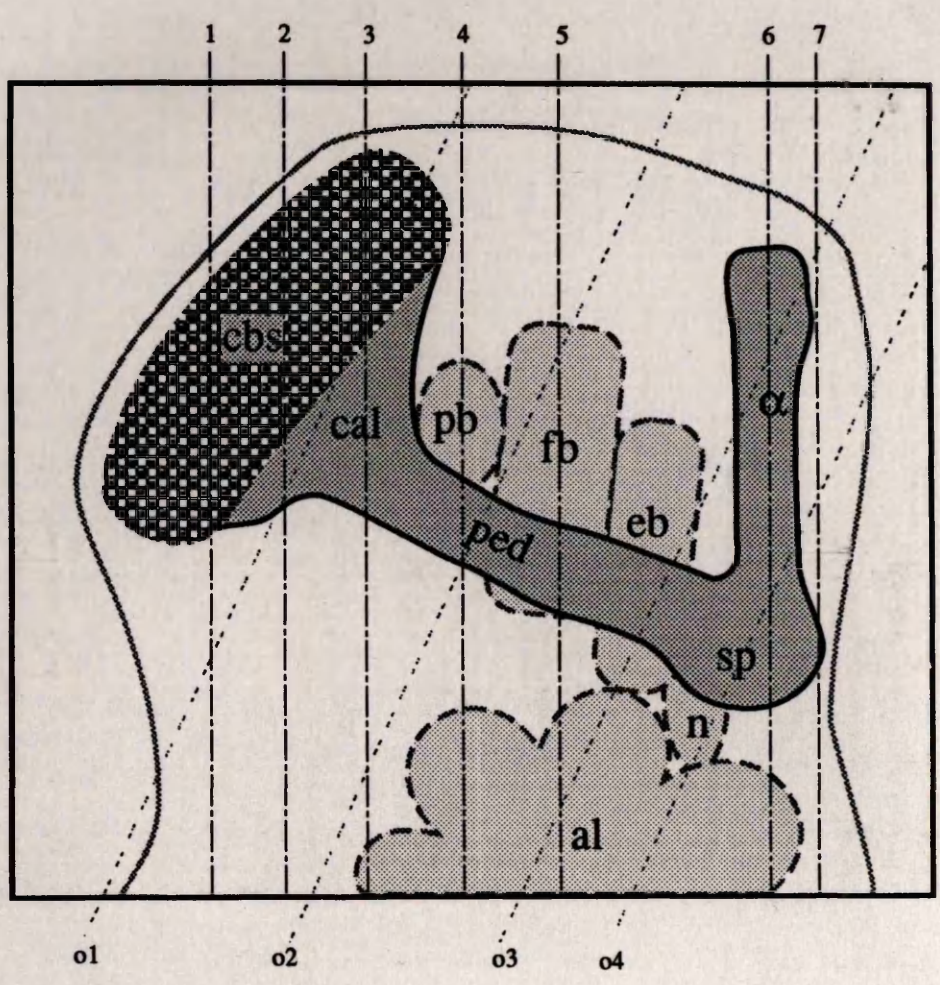


Fig 3.1 Confocal planes of sections

The saggital schematic representation is based on the one in Appendix 1. It indicates the approximate plane of section (1-7) of the following confocal sections. The faint oblique lines (o1-o4) represent the planes of section in fig 3.5. Indicated is a single mushroom body; cell body layer (cbs), calyx (cal), pedunculus (ped), spur (sp) and the α lobe (α). The following structures are also outlined: antennal lobes (al) and central complex; protocerebral bridge (pb), fan-shaped body (fb), ellipsoid body (eb), noduli (n).

All scale bars represent 10μm

Fig 3.2 Line 30Y: adult brain

Of all lines stained, 30Y has the strongest expression pattern. The lowest laser power and image intensification settings on the confocal were used to produce the sections above. A large number of neurons are revealed in this expression pattern, the predominant being within the mbs. The figure comprises four confocal sections showing the following. (a) A large number of densely packed Kenyon cell bodies (cbs), and a somewhat less brightly stained calyx (cal). (b) The pedunculus (ped) is strongly stained. (c) All three lobes are strongly stained (α, β and γ) as is the spur (sp). (d) This section at the anterior margin of the brain reveals the γ lobe.

Planes of section: (see fig 3.1) a = 2; b = 4; c = 6; d = 7

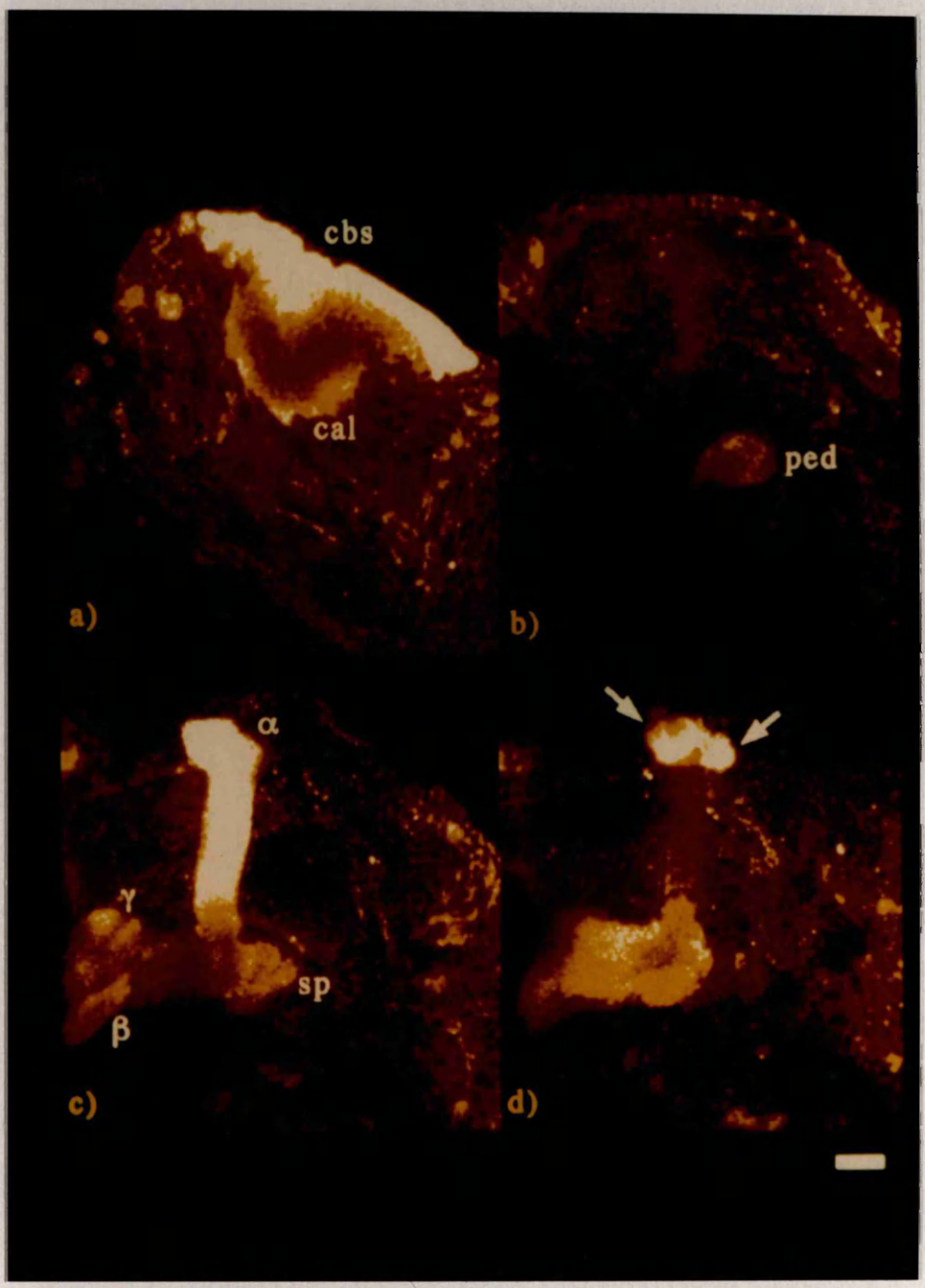

cbs
cal
a)
ped
b)
α
γ
sp
β
c)
d)

Fig 3.3 Line 201Y: adult brain

Confocal sections taken from P{GAL4} line 201Y. The sections on the left were taken from a younger individual (4-14 days), on the right, sections were taken from an older individual (>6 weeks). (a) and (b) Both show staining of the cell body regions. (c) This section through the pedunculus reveals a stained ring encompassing several narrow central tracts. (d) In the older individual the outer ring is stained, however the inner tracts are not labelled. (e) The spur (sp) and γ lobes stain strongly in the younger fly along with narrow components of the α (α) and β (β) lobes, possibly forming core elements of these lobes. (f) Only the spur (sp) and γ lobe are labelled in the older fly. (g) and (h) Both show the staining in the γ lobes.

Planes of section; (see fig 3.1) a,b = 2; c,d = 4; e,f = 6; g,h = 7

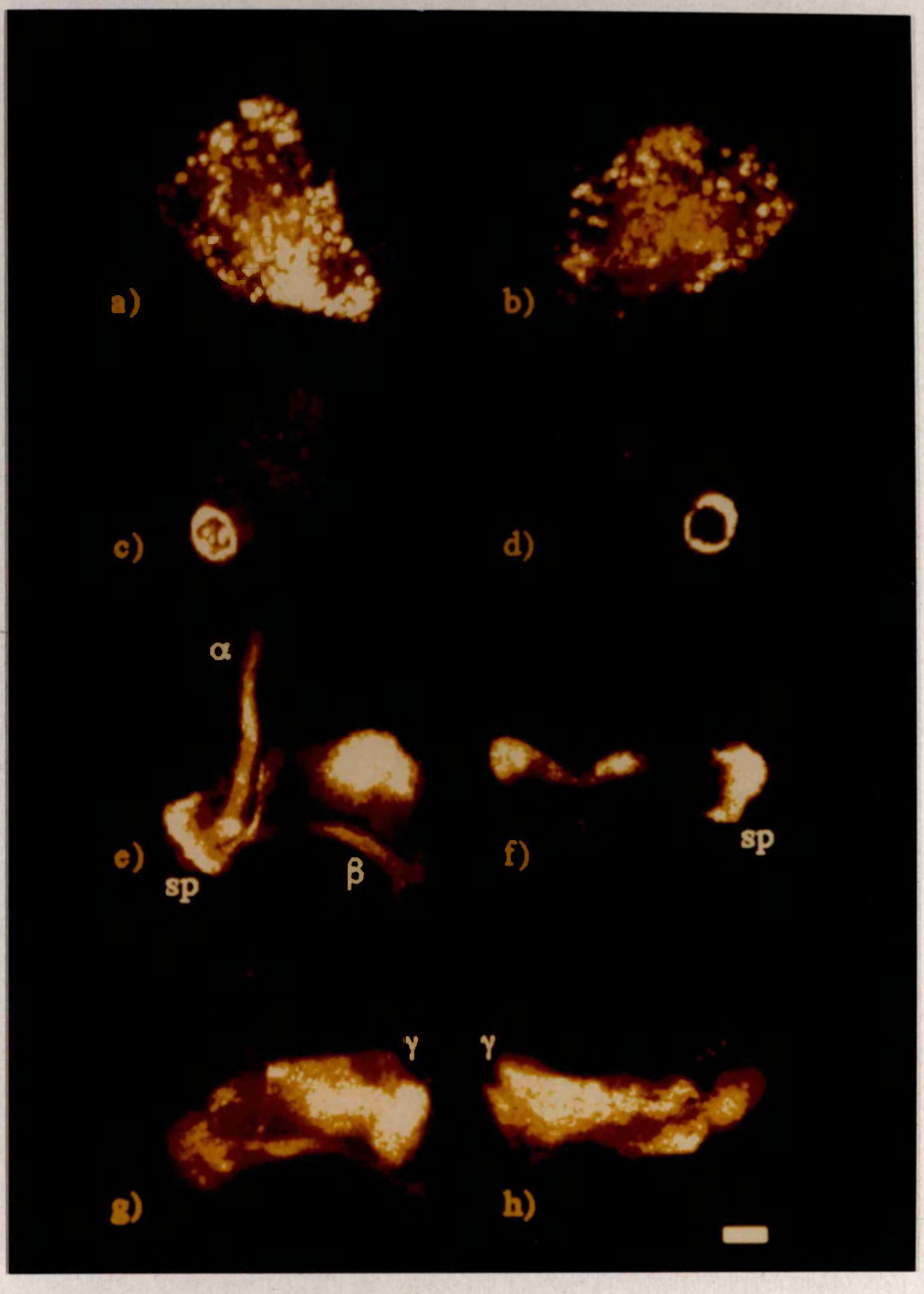

a)
b)
c)
d)
α
e)
sp
β
f)
sp
γ
γ
g)
h)

Fig 3.4 Line c739: adult brain

Confocal sections through P{GAL4} line c739. (a) Three of the four groups of Kenyon cell bodies (arrows) that the supply four tracts in the calyx that enter the pedunculus can be seen in this section. (b) In the pedunculus these four tracts fuse into two then subsequently fuse again into a single tract near the anterior end of the pedunculus. (c) Only the α and β lobes are stained in c739. Close examination reveals a very narrow unstained core region in both lobes. The spur and γ lobe are unstained. This pattern contrasts with that of 201Y.

Planes of section; (see fig 3.1) a = 2; b = 4; c = 6


a)
b)
α
c)
β

Fig 3.5 Line c772: adult brain

Confocal sections from line c772. (a) A large number of densely packed Kenyon cell bodies are revealed. (b) The outer region of the pedunculus is strongly stained. In the inner region, two tracts are unstained. (c) and (d) All three lobes stain in this line. The γ lobe and spur stain strongly. Of the α and β lobes, only the outer regions stain surrounding unstained core regions (arrows) in both. This brain is not exactly frontal thus the α and β lobes are revealed in different planes of section. Not all of the α lobe is revealed in any single section.

Planes of section; (see fig 3.1) a = o1; b = o2; c = o3; d = o4

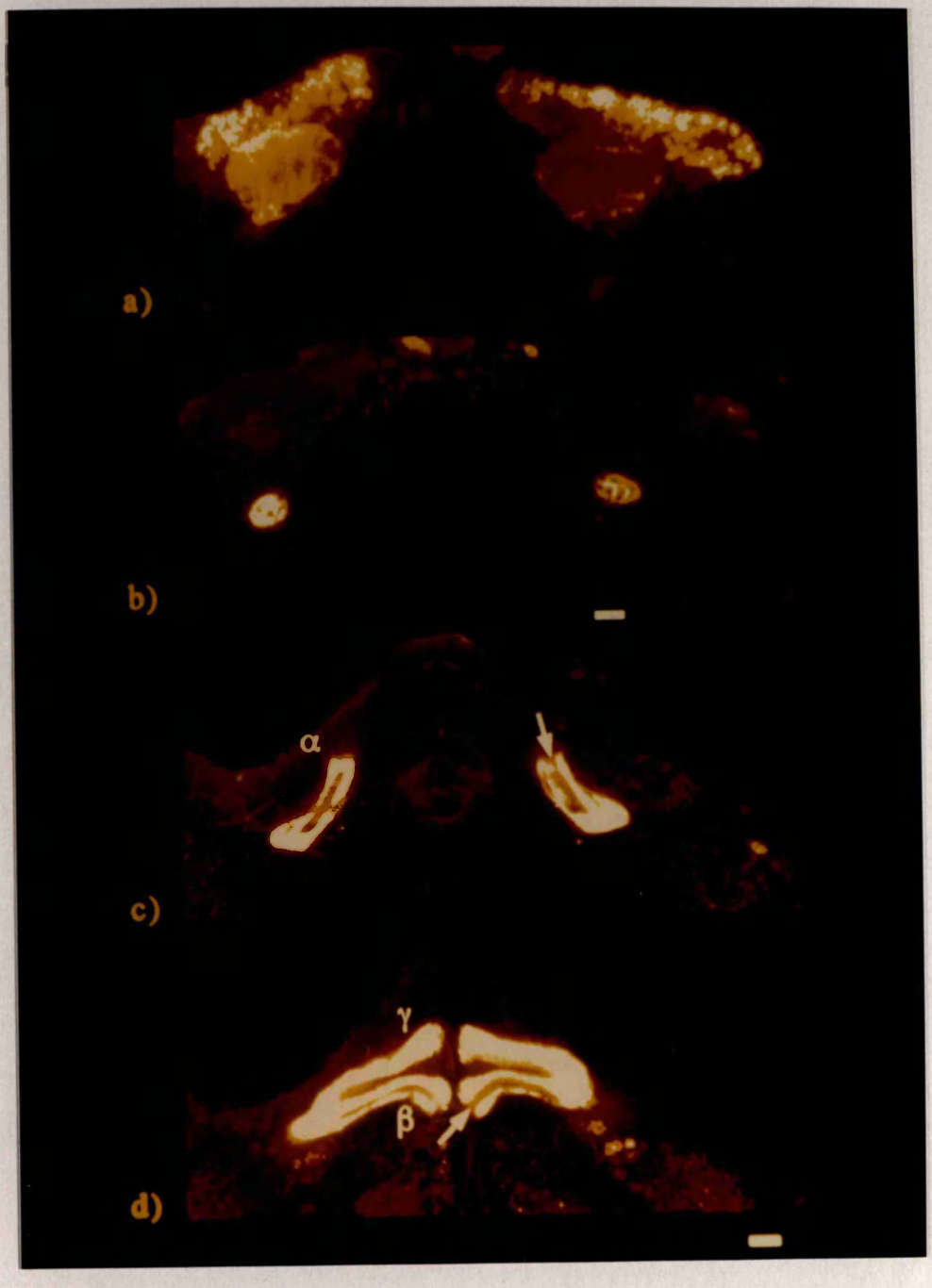

a)
b)
α
c)
γ
β
d)

Fig 3.6 Line c35: adult brain

Confocal sections from line c35. (a) A large number of evenly stained cell bodies are evident. (b) In the pedunculus there are two distinct staining intensities. The outer ring is less strongly stained than the inner region. (c) All three lobes and spur are stained, however the spur (sp) and γ lobes are less intensely stained than the α and β lobes.

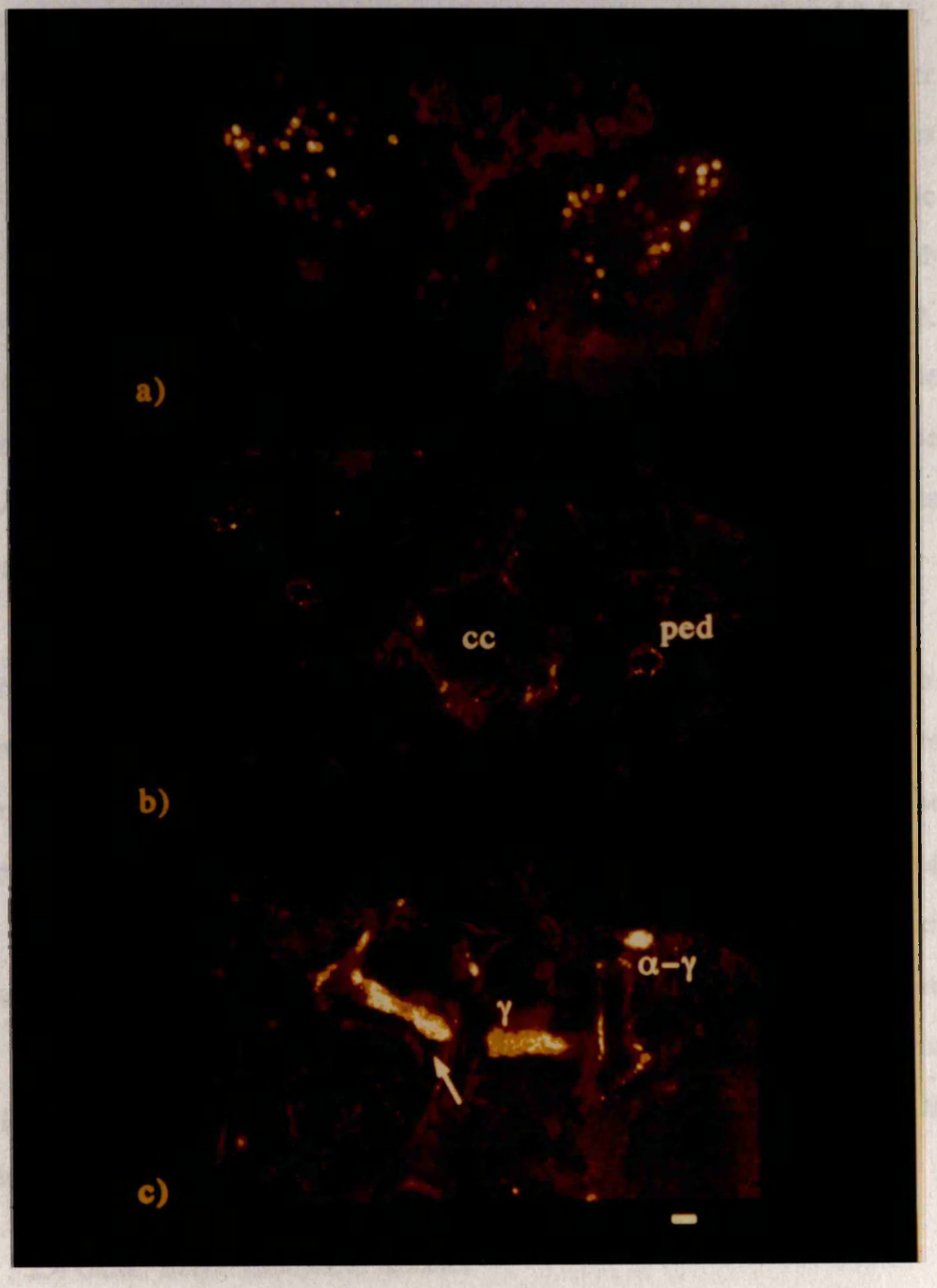
Planes of section; (see fig 3.1) a = 2; b = 5; c = 6


cal
a)
b)
α
γ
sp
β
c)

Fig 3.7 Line 259Y: adult brain

Frontal confocal sections though the mushroom bodies: (a) Only 40-50 scatted Kenyon cell bodies stain in this line and the calyx is likewise diffusely labelled, staining being predominantly along an outer margin. (b) A punctate ring of fibres is stained on the outer margin of the pedunculus. The central complex also stains in this line and can be seen between the pedunculus staining in this section. (c) The β lobe is unstained while staining of the α lobe is restricted to narrow strands. Staining of the γ lobe is restricted to a dorsal region. At this plane of section it is just dorsal to the unstained β lobe (arrow).

Planes of section; (see fig 3.1) a = 1; b = 5; c=6


a)
cc
ped
b)
α–γ
γ
c)

3.2.3 Other lines staining the mushroom bodies

All major structural features of the mushroom bodies revealed by the GAL4 expression patterns are most clearly demonstrated in the lines shown (figs 3.2-3.7). These expression patterns are summarised in schematic form in fig 3.8. Of the other lines, many had expression patterns overlapping to varying degrees with the ones described in detail. Although several of the lines were indistinguishable from the ones described here, most were significantly different. A few of the other mb patterns are described briefly in Chapter 5, Appendix 1 and Appendix 2. Chromosomal locations were available for most of the 31 lines and revealed with the exception of a single case, that all lines examined were unique insertions of the P{GAL4} element (Appendix 1). The two mb lines (c772 and c747) with the same chromosomal insertion point exhibited indistinguishable staining patterns.

Comparison between all 31 lines (summarised in table 3.1) revealed only one sub-structural feature not described above. The subdivision of the α and β lobes into inner and outer regions was found to be more complex. In many of the lines, the diameter of the staining within the α and β lobes differed from the lines described above. This suggested additional concentric subdivisions within those described. The α and β lobes are most likely comprised of many such concentric rings, at least four of which, can be readily identified by detailed analysis of expression patterns. Staining within these subdivisions was also reflected in the corresponding tracts in the pedunculus.

3.2.4 Other brain structures stained

All of the lines described here stain other regions of the brain, in addition to their mb staining. The pattern most restricted to the mbs is line 201Y. Lines c772, c35 and 30Y stain large numbers of neurons encompassing most neuropil regions. All the lines described above, stain a small number of secretory neurons, in the pars

intercerebralis (Strausfeld, N.J., 1976). The pars intercerebralis is visible in most P{GAL4} staining patterns irrespective of other staining in the brain (not shown). This staining may be similar to the larval salivary gland staining exhibited in most 3rd instar larvae (Brand, A. and Perrimon, N., 1993).

Table 3.1 Summary of the mushroom body staining patterns

The table opposite lists the major features of each P{GAL4} staining pattern analysed in this study. The *in situ* column lists the chromosomal location of the insert where known. The next two columns describe staining in the cell body region and calyx of the mushroom bodies. Strong describes patterns similar to that seen in 30, diffuse is similar though less instense. Clusters at the cell body region and split calyx are like that of line c739 where groups of cell bodies each seem to supply a separate unit of the calyx. The pedunculus staining is divided into outer and inner regions. Where tracts are distinct in the inner region, it has been noted. At the lobes, lines indicated as staining in a lobe system do not nessesarily staing the lobe in its entirity. Only lines where the staining in the lobe systems is very restricted is this indicated (eg 201Y and 259Y)

Line	*in situ*	cell bodies	calyx	outer ped	inner ped	alpha/beta lobe	spur + gamma
11Y	71C	clusters	split	none	stained	stained	none
17Y		diffuse	diffuse	stained	stained	stained	stained
30Y	70E	strong	strong	strong	strong	stained	stained
43Y	2C	clusters	split	none	stained	stained	extrinsic
45Y		clusters	split	none	stained	outer only	none
72Y	21B	strong	strong	stained	stained	stained	diffuse
103Y	2D	strong	strong	strong	strong	stained	stained
117Y	34C	clusters	clusters	none	stained	outer only	none
121Y	71B	clusters	split	none	tracts	stained	none
152Y		clusters	split	none	tracts	outer only	none
181Y	57B	diffuse	diffuse	stained	stained	stained	stained
201Y	56D	diffuse	diffuse	stained	tracts	inner only	stained
203Y	1C	strong	strong	stained	stained	outer only	stained
210Y	70B	diffuse	diffuse	none	tracts	mid region	none
227Y	1A	clusters	split	none	tracts	stained	none
252Y	42B	diffuse	diffuse	stained	stained	stained	stained
259Y	57F	diffuse	diffuse	partial	none	alpha-gamma	dorsal gamma
277Y	11B	clusters	split	none	tracts	stained	none
c35	44A	strong	strong	diffuse	tracts	stained	diffuse
c97		clusters	split	none	tracts	stained	none
c253	49D	strong	strong	stained	stained	stained	stained
c271	70C	strong	strong	stained	stained	stained	diffuse
c302	18C	clusters	split	none	tracts	outer only	none
c308a		diffuse	split	none	tracts	outer only	none
c309		strong	strong	stained	stained	stained	stained
c492b	3B	strong	strong	stained	stained	stained	stained
c737	49D	clusters	split	none	stained	stained	none
c739	40A	clusters	split	none	tracts	stained	none
c747	42A	strong	strong	stained	stained	outer only	stained
c772	42A	strong	strong	stained	stained	outer only	stained
c831	42B	strong	strong	stained	stained	stained	stained

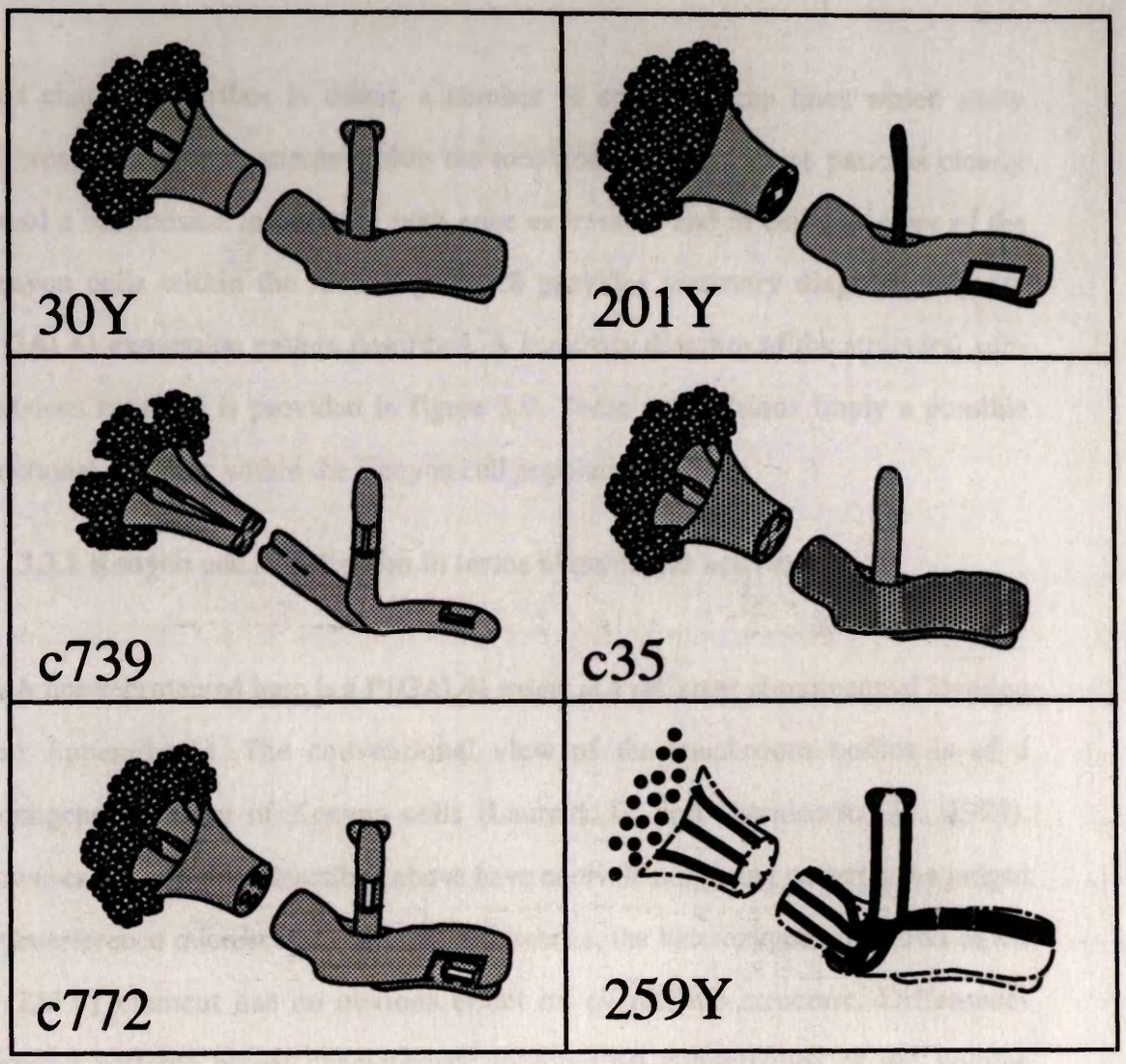


Fig 3.8 Summary outlines of P{GAL4} expression patterns in the mbs
Schematic representations of the P{GAL4} expression patterns as revealed by analysis of confocal reconstructions and confocal section series. The cell body region, calyx and initial region of the pedunculus have been separated from the lobes for clarity. Regions of the lobes and calyx have been cut away to reveal the deeper structure.

3.3 Discussion

This chapter describes in detail, a number of enhancer trap lines which show different expression patterns within the mushroom bodies. These patterns clearly reveal a subdivision in terms of both gene expression and in cell trajectory of the Kenyon cells within the mbs. Figure 3.8 provides summary diagrams of each P{GAL4} expression pattern described. A summary diagram of the structural sub-divisions revealed is provided in figure 3.9. These subdivisions imply a possible functional diversity within the Kenyon cell populations.

3.3.1 Kenyon cell sub-division in terms of enhancer activity

Each line represented here is a P{GAL4} insert at a different chromosomal location (see Appendix 1). The conventional view of the mushroom bodies is of a homogeneous array of Kenyon cells (Laurent, G. and Davidowitz, H., 1994). However, no two lines described above have equivalent staining patterns. As judged by interference microscopy and autofluorescence, the heterozygous insertion of the P{GAL4} element has no obvious effect on overall mb structure. Differences between patterns reveal characteristic longitudinal subdivisions of the volume encompassed by the whole mushroom body. With the exception of the extrinsic elements to the γ lobes noted in two of the lines (see Chapter 5), staining in all of the lines can be followed from the cell bodies, into the calyx, through the pedunculus and into the lobes.

3.3.2 Concentric sub-division of the lobes

The general pattern that emerges is of groups of axons at characteristic and often concentric positions within the pedunculus, that segregate to characteristic regions of the lobes. Reproducible differences of intensity help to distinguish parallel subdivisions, for example, the division of the α and β-lobe into inner and outer

components in lines 201Y (fig 3.3) and c772 (fig 3.5). Line c739 (fig 3.4) exemplifies the relationship between α and β lobes. The two lobes appear very similar in shape, size, and staining intensity within any one preparation.

When the α lobes stained in lines 30Y (figs 3.2) and c772 (fig 3.5) are compared with that in c739 (fig 3.4), there appears to be an extra component in the former. Two club-like 'gnarls' are present at the tip of the lobe in 30Y (arrows) and c772, one medial and one lateral. The expression pattern of line 259Y (fig 3.7) reveals two narrow, outer strands in the α lobe that extend into the lateral and medial 'gnarls'. In this line, the β lobes are unstained as is the inner region of the pedunculus. A dorsal region of γ is stained, as is the outer region of the pedunculus, suggesting a relationship between the α staining in this line with γ lobe staining rather than β lobe staining. This component of the α lobe has been referred to here as the α/γ lobe. In all other lines, the 'gnarls' are only stained in lines that also stain the γ lobe. A spur-like structure occurs at the branch point of the pedunculus and lobes. Within any one line, the intensity of the spur corresponds to that of the γ-lobe indicating that these are related.

Such intensity variations within components of the structure could either be due to different levels of reporter expression or differences in density of axons staining. Lines such as c35 (fig 3.6) show variations in intensity of staining at the pedunculus and lobes. However, the cell bodies within any one line show little or no variation in staining intensity, thus the differences are more likely to arise from density of stained fibres (ratio between unstained and stained fibres in any one region) rather than from different levels of reporter expression.

3.3.3 Subdivisions in the pedunculus relate to those seen in the lobes

Subdivisions in the pedunculus can be traced directly to the lobes. In line 201Y (fig 3.3) where the central cores of α and β are staining, the staining in these lobes can be traced back to four individual tracts entering the pedunculus from the calyx (fig 3.10). Although these four tracts rapidly fuse to form two tracts they can also be seen in lines staining the outer components of α and β as in lines c35 (fig 3.6) and c739 (fig 3.5).

The staining pattern within any one line was invariably consistent between individuals with the exception of line 201Y. The α and β lobe staining is lost in some older 201Y individuals over 6 weeks (fig 3.3). In these flies, staining can be seen in the γ lobe, spur and an outer ring of the pedunculus. The staining normally visible in the α and β lobes, and in the inner region of the pedunculus, is missing. This suggests that the remaining γ lobe staining represents a Kenyon cell type that projects along the outer rim of the pedunculus to the spur and γ lobe alone. The staining lost in these older individuals probably represents a Kenyon cell type that projects along the inner region of the pedunculus to the α and β lobes.

The γ lobe is not concentrically subdivided into inner and outer 'rings' like the α and β lobes. The γ lobe is also subdivided, though the exact nature of the subdivisions remains unclear. There are differences between the extent of γ staining in lines c35 (fig 3.6) , 201Y (fig 3.3) and line 259Y (fig 3.7). Staining in the γ lobe is invariably associated with staining in the spur and in the outer ring of the pedunculus surrounding the area associated with the α and β lobes. The staining pattern of line 259Y provides evidence for a cell type which projects into both the γ lobe and α lobe. In particular a lateral bulb at the tip of the α lobe appears associated with staining in the γ lobe. As this may be distinct from the α and β lobes, the term α-γ has been applied to this lobe (fig 3.8).

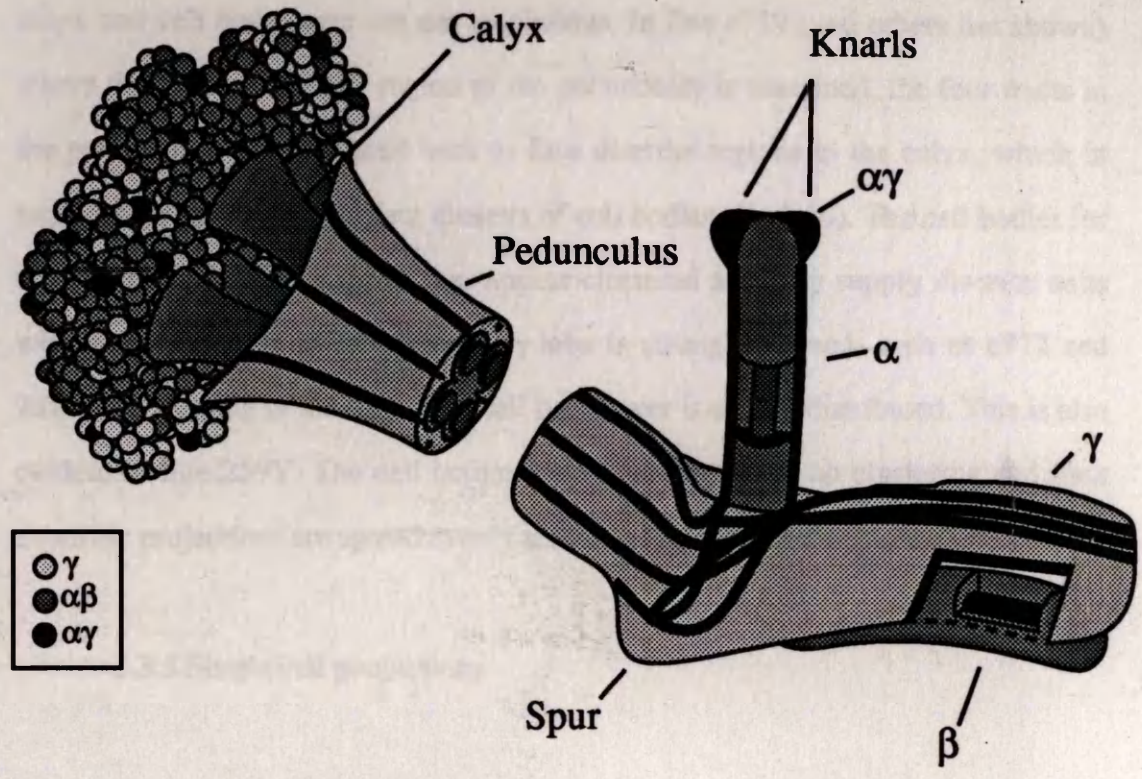


Fig 3.9 Schematic representation of mushroom body substructure

The illustration summarises the major mushroom body structural features identified from P{GAL4} expression patterns. Continuous substructure is shaded appropriately. The cell body region, calyx and initial region of the pedunculus have been separated from the lobes for clarity. Regions of the lobes and calyx have been cut away to reveal the deeper structure. The approximate locations for the Kenyon cell bodies that correspond to the subdivisions in the pedunculus and lobes have been indicated by shading them appropriately.

3.3.4 Subdivision in the calyx and cell body layer

In contrast to the divisions observed in the lobes and pedunculus, divisions of the calyx and cell body layer are not as obvious. In line c739 (and others not shown) where the γ lobe and outer region of the pedunculus is unstained, the four tracts in the pedunculus can be traced back to four discrete regions in the calyx, which in turn, can be traced back to four clusters of cell bodies (fig 3.10). The cell bodies for the α and β lobe fibres sometimes appear clustered and may supply discrete units within the calyx. In lines where the γ lobe is strongly stained, such as c772 and 201Y, the staining in the calyx and cell body layer is evenly distributed. This is also evident in line 259Y. The cell bodies for the γ lobes show no clustering and their dendritic projections are spread evenly across the whole calyx volume.

3.3.5 Single cell projections

From the observations described, above it is possible to predict a number of cell types within the Kenyon cell population. Clearly, there are Kenyon cells whose fibres project along a central component of the pedunculus into the α and β lobes. There are also cell types which project to the γ lobe, via the spur and outer component of the pedunculus. Some of these have projections into γ lobe alone and some also project into the α-γ region.

Prior to this study, two populations of Kenyon cells had been described, based upon their axonal diameter within the pedunculus (Heisenberg, M., 1980). Narrow fibres in the outer region of the pedunculus continuous with the α and β lobes were suggested, with the outer, broader fibres continuous with the spur and γ lobe. This study confirms this, and suggests a much higher degree of subdivision.

Fig 3.10 3D Reconstruction of mushroom body expression patterns

Two 3D reconstructions of single mushroom bodies that reveal specific aspects of the structure. Pseudocolour in the images highlight regional intensity differences. (upper) Medial and dorsal 3D representation of the pedunculus and lobes in line 201Y. The intensity difference between the α/β fibres and the γ fibres in the pedunculus is slightly different thus the α/β fibres are coloured yellow and the γ fibres red. The four tracts in the pedunculus are clear. (lower) Lateral and dorsal 3D representation a whole mb in line c739 showing 3 of the 4 calyx subdivisions (arrow) and clustering of the cell bodies (black dots). The other calyx subdivision and cluster of cell bodies are obscured.


ped
β
γ
α

3.4 Summary

Confocal microscopy of enhancer-trap expression patterns provides high resolution 3D images that can reveal neuronal structure. Analysis of thirty one lines that stain in the mushroom bodies has provided evidence suggesting a degree of structural subdivision. Rather that being comprised of a homogenous population of intrinsic cells, the mushroom bodies exhibit a degree of structural heterogeneity. Continuity of staining pattern along different regions of the mb structure allows definition of three different Kenyon cell projection patterns, and suggests many more. The gross structural features of the mushroom bodies are also revealed to be subdivided with respect to enhancer activity.

Chapter 4. Pupal Development of the Mushroom Bodies

4.1 Introduction

The gross development of the *Drosophila* mushroom bodies has been studied by classical histological techniques (Ito, K., 1990, Ito, K. and Hotta, Y., 1992). A structure that can be recognised as being a mushroom body by presence of cell body layer, calyx, pedunculus and lobe system, was described prior to larval hatching. Mushroom bodies remain visible throughout all subsequent developmental stages. During metamorphosis, many of the larval organs and tissues regenerate and are replaced by adult specific ones (Bainbridge, S.P. and Bownes, M., 1981). The mushroom bodies remain throughout metamorphosis and are enlarged during this period. Analysis of fibre number from late larval stages into early adult life, reveals a sharp drop in fibre number early in pupation followed, by an increase a few hours later (fig 1.4) (Technau, G.M. and Heisenberg, M., 1982). An attempt at counting cell bodies suggested that the total number of bodies continued to rise throughout this period. This implied a degeneration of fibres followed by a regrowth of new fibres from the mb cell bodies. A small central group of fibres (400) within the pedunculus were proposed to act as guides for the new and regenerating fibres. This chapter describes an attempt to unravel the developmental process that leads to the structural characteristics of the mushroom bodies discussed in the previous chapter.

A selection of P{GAL4} lines whose expression patterns in the adult brain reveal structural features of the mbs (see Chapter 3) were examined at the third instar larval stage. The larval mushroom bodies were easily recognised, as the overall shape exhibits many of the gross structural features of the adult structure (Ito, K., 1990). Lines exhibiting expression within the larval mushroom bodies were subsequently

examined at stages throughout pupation, from the immobile pre-pupal phase onwards, and at regular intervals, for a further 48 hours. The lines examined were 201Y, c739, 203Y, 30Y and c772. As these lines show reporter expression in other brain neuropil, a selection of stages from lines c133 (antennal lobes and AGT) and c161 (central complex) were also examined. Obviously, changes in patterns observed throughout development may be due to gene expression changes rather than any structural changes. A number of individuals were selected at four hourly intervals to detect any rapid change in enhancer activity. A slow change in enhancer activity within any population of Kenyon cells cannot be discounted entirely for any of the lines.

4.2 P{GAL4} expression in the larval mushroom bodies

4.2.1 The Lobes of the Larval Mushroom Bodies

With the exception of P{GAL4} line c772, all the mb lines exhibited expression patterns within the larval mbs (fig 4.1). The larval mushroom bodies are outwardly similar to the those of the adult, both having a cell body layer, calyx, pedunculus, and lobe system. However, close examination of the expression patterns revealed some striking differences. No equivalent of the adult γ lobe was observed. The pedunculus bifurcated into two lobes. In all lines, two lobes were stained, a single medially projecting lobe and another perpendicular lobe which resembled the α and β lobe system of the adult. A small lateral projection was observed at the bifurcation of the pedunculus. In line c772, mushroom body staining was not visible until late in development (just prior to hatching).

Fig 4.1 P{GAL4} expression in the larval mbs

Confocal reconstructions of P{GAL4} expression within the larval mbs. The term 'vertical' as used in the following descriptions relate to the sections themselves not the larvae as a whole. (a) Reconstruction of the expression pattern in line 30Y, probably the most extensively staining mb line although the extent of staining in surrounding neuropil obscures reconstructions of the entire mb structure. Two lobes are evident; one vertically projecting (v) with a slight out-swelling near its tip. The medially projecting lobe (m) shows a much larger out-swelling. (b) 201Y however, is very specific in its staining in the larval brain as well as in the adult. Like 30Y its lobes show out-swellings at the tips (arrows) however, in each a central core region is unstained. (c) c739 appears somewhat different with much narrower component staining than either 30Y or 201Y probably staining a central core (possibly the corresponding to the unstained core in line 201Y). The large protuberances near the tips of the lobes (arrow) may well account for the out-swelling seen in the previous two lines. (d) Line 203Y like that of 30Y stains a large amount of surrounding neuropil and thus it is difficult to reconstruct the whole mb without obscuring it. The reconstruction of the larval lobes reveals what resembles 201Y in shape but much of the inner structure is unstained and it appears to stain a small number of fibres supplying a diffuse network in the outer region of the lobes.

a)
v
m
b)
c)
d)

The orientation of the larval mushroom bodies is somewhat different from the adult. The adult brains are tilted through 90° with respect to the larval brain. The lobes of the adult mb lie in a frontal plane and the pedunculus projects approximately horizontally to the posterior region of the brain. In the larval brain, the lobes of the mushroom body lie in the horizontal plane and the pedunculus projects along a dorsal/ventral plane. As the exact orientation of the brain is not really important here, the larval brain is presented in a similar way to the description of the adult patterns in Chapter 3.

4.2.2 Nomenclature used for description of the larval mushroom bodies

As the larval lobes are clearly different in outward appearance from those of the adult, different nomenclature has been used to describe them. The vertically (see above) projecting lobe is described here as the 'vertical lobe', while the medially projecting lobe, is described as the 'medial lobe' (fig 4.2). With respect to the pedunculus, calyx, and cell body layer, the larval structures closely resemble those of the adult and the nomenclature remains unaltered.

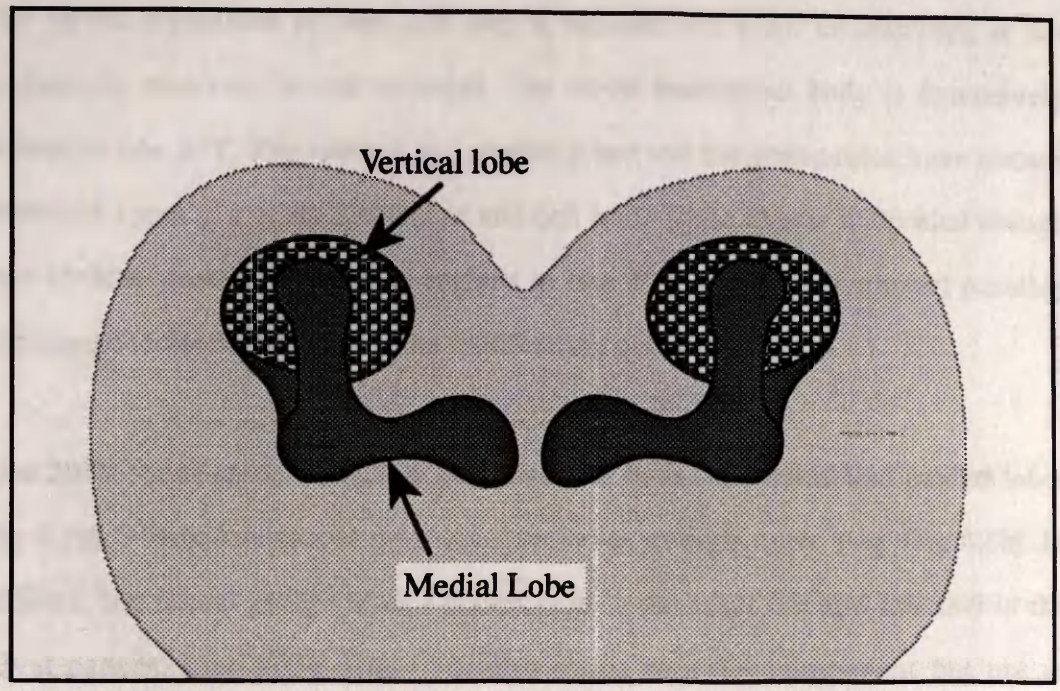


Fig 4.2 Nomenclature used for larval mbs

This simple diagram demonstrates the nomenclature used here for the lobes of the mbs in the 3rd instar larval stage.

4.2.3 Expression patterns in the larval mb lobes in comparison with the adult patterns

The larval expression of line 30Y (fig 4.1a), like it's adult counterpart, is not exclusively restricted to mb neuropil. The larval mushroom body is extensively stained in line 30Y. The vertical and medial lobes and the pedunculus have narrow unstained cores (fig 4.3a). The calyx and cell body layer appear undivided though four obvious unstained spherical regions in line 30Y (8-10 μm diameter) possibly correspond to the neuroblasts of the proliferation quartet (fig 4.4a).

Line 201Y stains an outer, hollow component of both the vertical and medial lobes (fig 4.1b). The pedunculus is similarly revealed as a single outer 'ring' (fig 4.3b). In addition, the lateral groups of cell bodies seen in the adult are also labelled in the larval pattern. Line 203Y (fig 4.1d) also stains an outer component but not as complete as that observed in line 201Y. Line c739 (fig 4.1c) stains a much narrower, presumably inner component of the vertical and medial lobes. The pedunculus is correspondingly much narrower but a narrow unstained core remains (fig 4.3c). The calyx however can be seen split into four tracts and the cell bodies clustered into four discrete groups (fig 4.4b).

Fig 4.3 Cross sections through the pedunculus of 3rd instar larval mbs

Cross sections though a mid region of the pedunculus. (a) 30Y stains an outer ring-like region with an unstained core. (b) 201Y staining is restricted to an outer ring. (c) line c739 stains a narrower ring than that observed in lines 201Y or 30Y with a very narrow unstained core

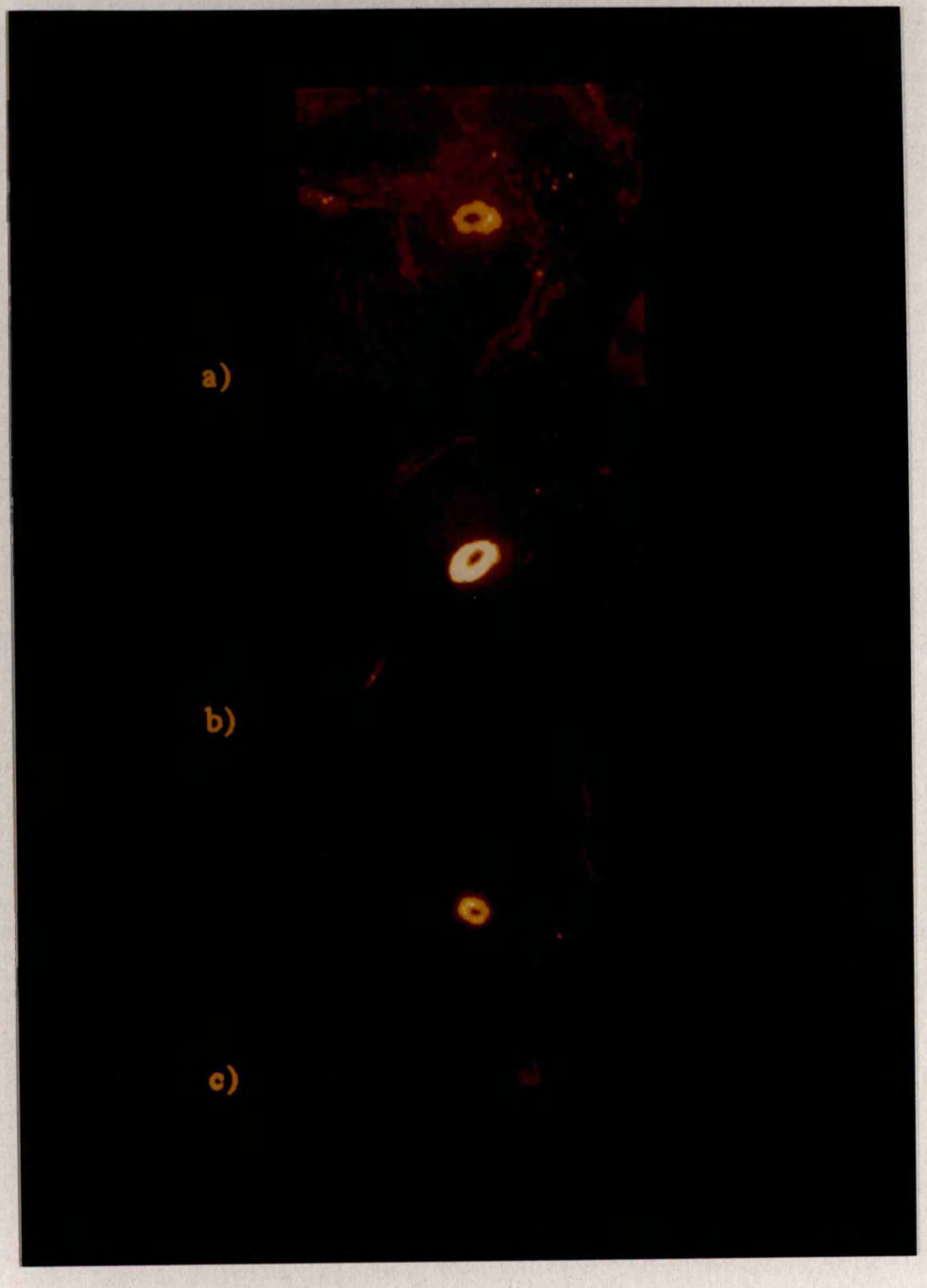
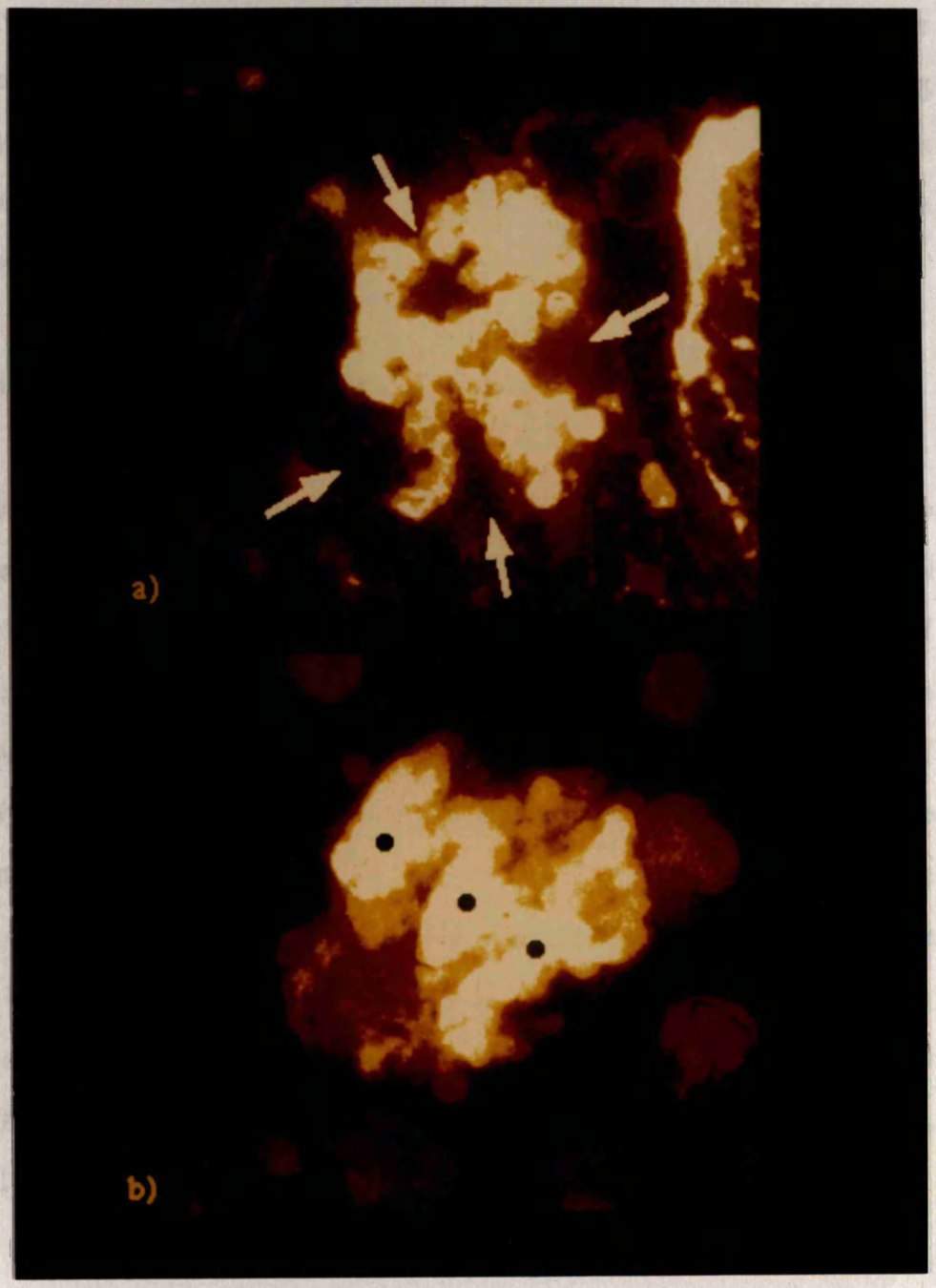

a)
b)
c)

Fig 4.4 Cross sections through the cell body layer of 3rd instar larval mbs

Two confocal sections through the cell body layers of lines 30Y (upper) and c739 (lower). In line 30Y, large, roughly spherical unstained regions within the cell body cluster most probably correspond to the neuroblasts of the proliferation quartet (arrows). Three of the four cell body clusters in line c739 (lower) can be seen clearly at this plane of section (dark filled circles).


a)
b)

4.3 Pupal development of the mushroom bodies revealed by P{GAL4} expression patterns

Each larval staining pattern was examined over the first two days of pupation where several developmental stages became apparent (figs 4.5-4.8). Little happens during the early stages of pupal development. After this initial inactive period, the lobes appear to degenerate in most of the lines. Again this is followed by a brief inactive period preceding formation of the adult structures.

4.3.1 Fibre degeneration in the larval vertical and medial lobes

The larval structure is maintained throughout the initial pupal stages, from the white pre-pupal stage for a period of around 8 hours. After this time, staining in the larval lobes is lost with the vertical lobe degenerating completely in all lines except 30Y where a reduced vertical component is visible throughout all stages of pupation (figs 4.7). However, a remnant of the medial lobe remains in all lines, largest in line 30Y (fig 4.7b). Loss of staining in the lobes is complete by around 20 hours, though some fragments of lobes are stained in the occasional individual after 24 hours. The staining intensity of the pedunculus is much less than that observed in either the adult or larval stages. This lowered intensity in the pedunculus suggests a smaller number of fibres is stained. Following the loss of staining in the lobes, presumably due to fibre degeneration, little appears to happen until formation of the adult lobes, some 8-12 hours later.

4.3.2 Formation of the adult lobes

The adult lobes begin to form at around 32-36 hours. Firstly the γ lobe appears in all lines and appears to grow out from the reduced medial lobe. Interestingly, in line c739 (fig 4.6) where the γ lobe does not stain in the adult (fig 3.4), it is visible at this

stage of pupation. However, this is a brief stage as, almost as soon as the γ becomes visible in line c739, it begins to disappear and is lost by around 48 hours. In the case of line 201Y (fig 4.5), the core elements of α and β that normally stain in the adult (fig 3.3) do not form immediately. A few faint fibres are present in some brains at around 28-32 hours but are not normally visible until after approximately 36 hours. Correspondingly, the α and β lobes that form in line 203Y (fig 4.8) initially show no evidence of an unstained core region at 24 hours. As the lobe broadens, a hollow central region emerges, suggesting new fibre growth at the centre of the pedunculus and lobes. The older fibres are thus displaced towards the periphery of the pedunculus and lobes.

Fig 4.5 Line 201Y: Pupal developmental series

The timed series of line 201Y is particularly informative primarily due to the restricted expression pattern of line 201Y in the mushroom bodies. The initial larval pattern is more or less maintained throughout the early pupal stages. After 8hrs or so, the vertical lobe staining starts to reduce in both size and intensity, more or less disappearing completely within a four hour period. The medial lobe degenerates during the same period but not to such a great extent. (a) After 12 hours, no vertical lobe is present, and the medial lobe in much reduced in size and intensity. The pedunculus itself appears unchanged. Little happens for a further twenty hours after which, the medial lobe elongates and broadens into a lobe that closely resembles the adult γ lobe. (b) 'γ' lobe formation appears complete after 36 hours. (c) Within four to eight hours of the γ lobe formation, narrow α and β lobes appear. In this section, the α and β lobes are very faint. (d) The α and β lobes intensify to the resemble the complete adult pattern within a further 24 hours. The central group of cell bodies are those of the pars intercerebralis (pars) which is stained in most P{GAL4} lines.

a)
b)
pars
c)
d)

Fig 4.6 Line c739: Pupal developmental series

Line c739, in many ways the converse of line 201Y with respect to both it's larval and adult mb expression patterns, stains a set of larval fibres that are most likely enclosed by the fibres stained in line 201Y. Like 201Y, the larval pattern remains for the most part unchanged for the first few hours. (a) Between eight and sixteen hours after puparium formation the vertical lobe staining disappears. A short component of the medial lobe remains and, in addition, a small anterior projection forms. Little changes for some 20 hours after which, all three 'adult' lobes form. (b) At 36 hours, lobes that very closely resemble the adult α, β and γ lobes are all stained. (c) Over the next 12 hours, the staining in the γ lobe is lost and only the α and β similar to that observed in the adult remains.

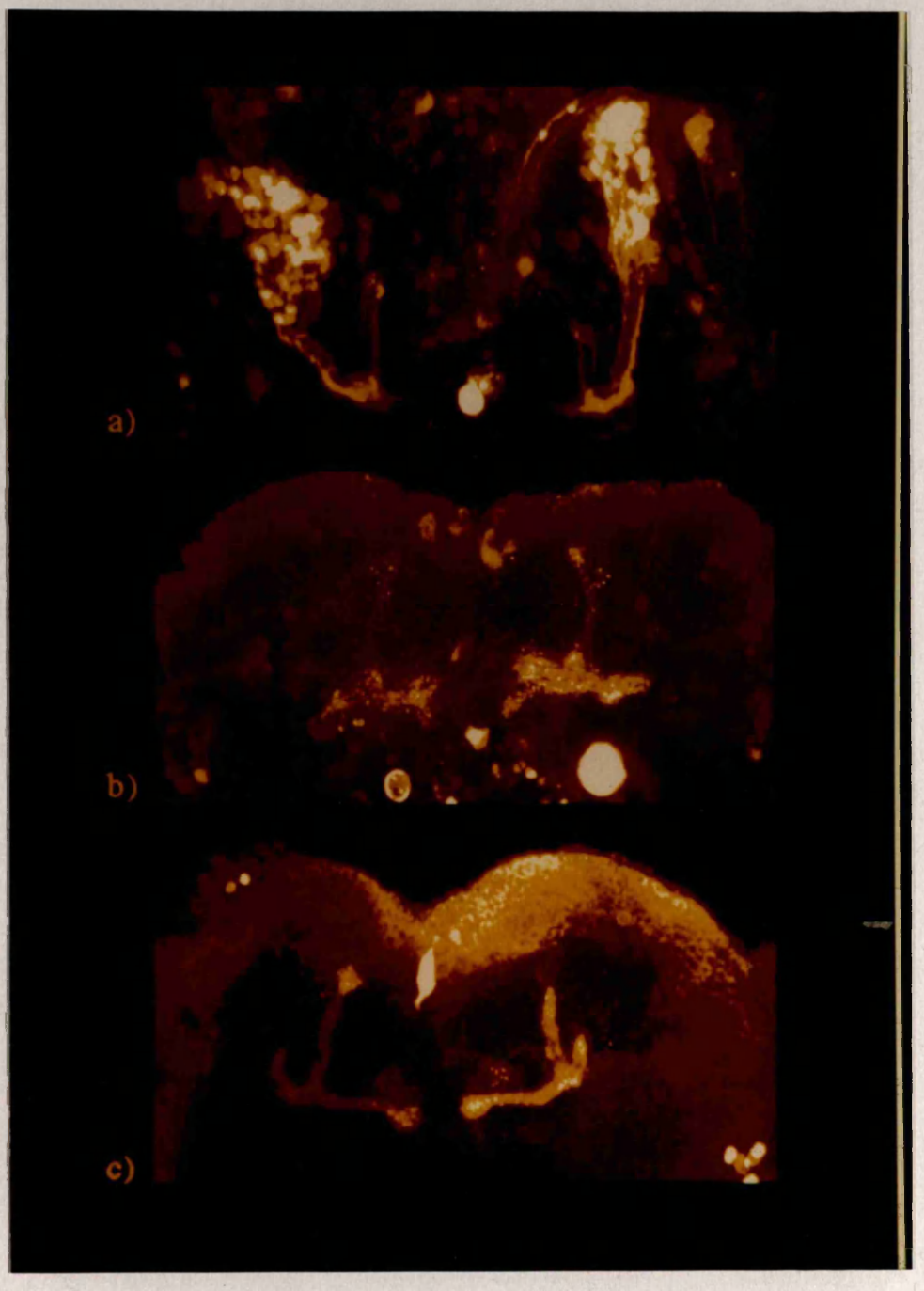

a)
b)
c)

Fig 4.7 Line 30Y: Pupal developmental series

Line 30Y, although heavily staining the mbs at all stages of development is harder to visualise as a result of the intensity of staining in surrounding neuropil. (a) Between 8 and 20 hours the lobe staining is reduced but not lost entirely in either lobe.. The medial lobe staining remains although reduced in size from the larval pattern. (b) At 30 hours, the γ starts to become visible with the still reduced vertical and medial lobes. (c) By 36 hours, the pattern is almost complete with three lobes closely resembling the adult pattern.

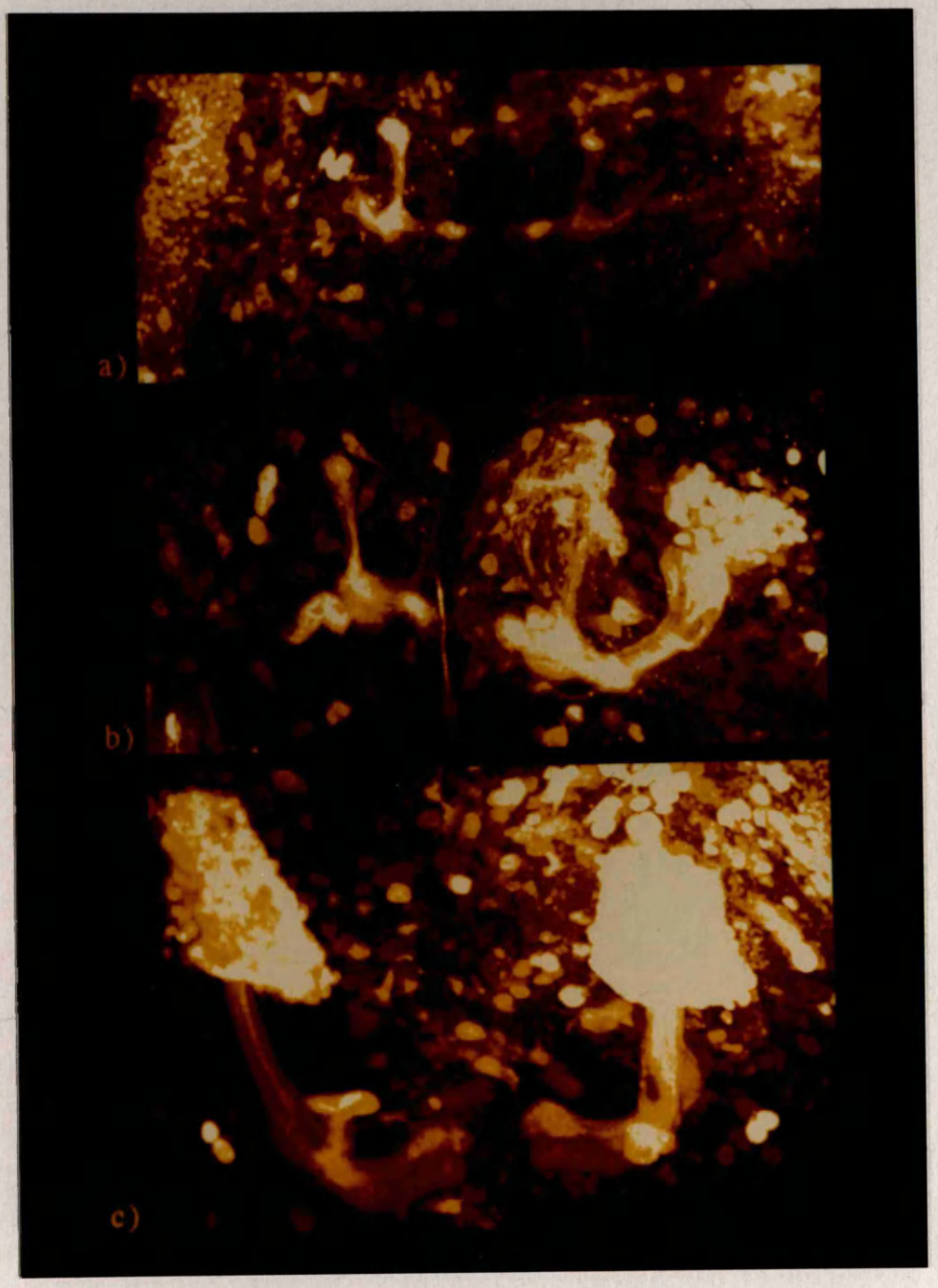
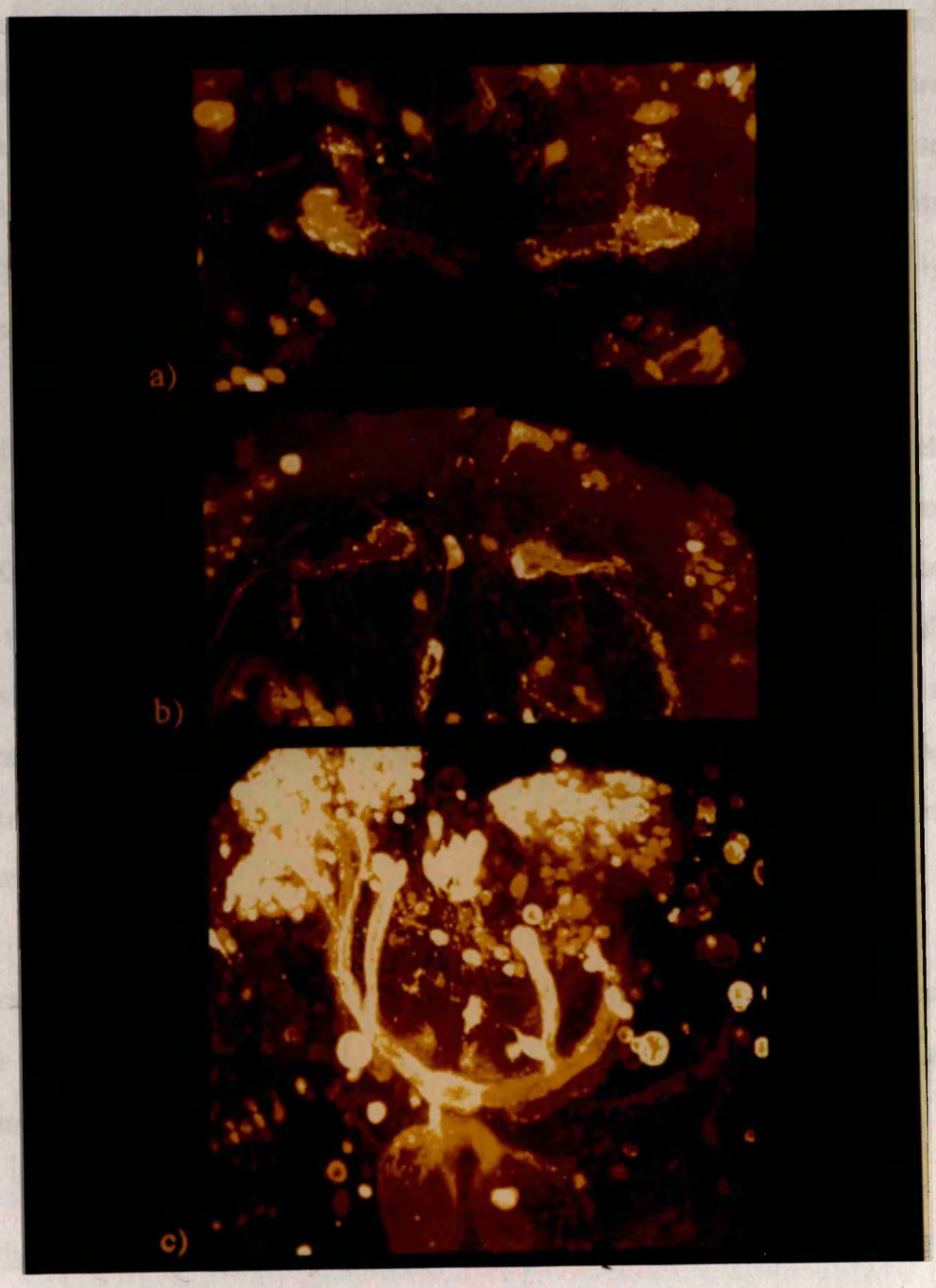

a)
b)
c)

Fig 4.8 Line 203Y: Pupal developmental series

The larval staining pattern of line 203Y resembles that seen in 201Y in that outer components of the vertical and medial lobes can be seen. However, the staining is not as intense and close examination reveals what appears to be a diffuse network of fibres within an outer shell of the lobe (i.e. less fibres rather than lowered levels of reporter expression). (a) The initial development from essentially the larval pattern of this line is more or less similar to that seen in the other lines with the vertical and medial lobes degenerating quickly after the 12 hour preparation illustrated. (b) At 20 hours, only a trace of the medial lobe remains. (c) The adult structure looks complete at 50 hours with hollow α and β lobes and a fully formed γ. Note the antennal lobe staining below the mushroom bodies.


a)
b)
c)

4.4 Developmental staining in other brain regions

Lines c133 (antennal lobes) and c161 (central complex) were examined at a selection of developmental stages. Line c133 stains a subset of dorsal and lateral antennal glomeruli in the adult (see Chapter 5, fig 5.5). A subset of AGT fibres are also stained. In the larval brain, three small spherical structures were stained. These were in a region corresponding with the antennal lobes and sent projections towards the calyx of the mushroom bodies. Throughout development, this structure slowly grew in size and complexity into the normal adult expression pattern.

Line c161 (not shown) stains a subset of central neurons. Approximately twenty cell bodies located at the posterior, dorsal margin of the brain, medial to the mushroom body Kenyon cell bodies, are stained in each hemisphere. These cell bodies send fibres which send fibres to the protocerebral bridge, ellipsoid body and noduli (Hanesch, U., et al., 1989). In the larval brain, several unidentified structures, presumably unrelated to the central complex, are stained. During pupation, at around 36 hours, a few faint fibres become visible in the ellipsoid body, and after a further 12 hours, the noduli then the protocerebral bridge become visible.

The gradual changes in expression patterns observed in these lines suggest that similar rates of changes in the mushroom body expression patterns are due to structural reorganisation rather than changes in gene expression.

4.5 Discussion

4.5.1 Parallel subdivision in the larval mushroom bodies

The lobes of the larval mushroom bodies, like their adult counterparts, show a concentric organisation revealed by the P{GAL4} expression patterns. However,

these patterns are quite distinct from those seen in the adult. Most obvious, is the lack of any γ-lobe like structure. As the larval mb lobes are obviously different, at least with respect to their outward geometry, an alternative nomenclature would appear to be required. As the developmental analysis shows, the relationship between the adult lobes and the larval ones is probably less close than expected; to use α or β in describing the larval lobes would most likely be incorrect. Both the vertical and medial lobes show inner and outer subdivisions. Lines 30Y and 201Y reveal the outer shape of the larval lobes. The unstained core in line 201Y may correspond with the narrow structure stained in line c739. Line 203Y shows a further subdivision, with an extreme outer region stained. In all lines examined, there is an, albeit very narrow, unstained core in the pedunculus the nature of which is unknown.

The subdivision of the larval pedunculus is simpler than that seen in the adult expression patterns. There is no evidence for the four central tracts seen in the adult. In the larvae, the pedunculi subdivisions appear to be a simple concentric arrangement of rings. Like the lobes, an unstained core region is present in all the lines.

Whether the differences between the larval mb expression patterns and those described in the adult are a result of structural re-organisation or changes in gene expression, can only be addressed by close examination of the developing structure through pupation.

4.5.2 Degeneration of the larval structure

Much of the lobes of the larval mbs can be seen to degenerate, at least back to the level of the bifurcation of the pedunculus. This change can be observed in all the lines examined, though the changes are less radical in line 30Y. All lines exhibit a

similar change in staining pattern suggesting axonal degeneration rather than a temporal change in gene expression. The level of degeneration is however, difficult to judge precisely. The neurons may be completely re-grown and the pedunculus staining thus represents an equilibrium between new outgrowing adult fibres and older degenerating larval fibres. However, an evenly stained remnant of the pedunculus is present in all lines for a considerable period after initial degeneration suggesting that at least some of the fibres may be present throughout. A group of fibres that is present in the pedunculus throughout metamorphosis has been described previously (Technau, G.M. and Heisenberg, M., 1982) It was suggested that they were a centrally located bundle. However, the fibres described here are not central but rather, those of an outer ring. In fact all lines examined had an unstained core region in the pedunculus which may be analogous to the dense core of fibres described previously.

4.5.3 Formation of the adult γ lobe

The γ lobe first appears in preparations at around 30 hours. The α lobe may be forming in a number of ways. The adult γ lobe appears to grow out from the remnant of the larval medial lobe. It is also possible that fibres from the lateral projection, in the larva, form part of the adult structure. The lobe may be formed from a combination of fibres running to both larval structures. The γ lobe stains briefly in line c739, though it is impossible to determine whether this is a brief activation of gene expression or the emergence of a small number of short lived fibres. The fibres which survive in the larval vertical lobe of line 30Y may represent the α-γ fibres in the adult.

4.5.4 Formation of the adult α and β lobes

The α and β lobes form just after the γ lobe with the exception of the subset of fibres which survive from the larval pattern in line 30Y. The lobes appear narrow at first

and in line 203Y, the core region is stained whereas in the adult, the lobes have an unstained core region. As the lobes develop further, the stained region broadens and, in line 203Y, an unstained core region emerges. At the same time, the first traces of the narrow core fibres that stain in line 201Y emerge. The appearance of the α and β lobes is mirrored by staining in the core regions of the pedunculus. It appears that the α and β lobes form at least mostly, and possibly entirely, during the pupal phase.

4.5.5 Relationship between the proliferation quartet and the developing mb.

The proliferation quartet of neuroblasts that give rise to the mb Kenyon cells are active from the embryo until late pupal stages (fig 1.3). In line c739, the four fold symmetry observed in the α and β lobe fibres at the level of the pedunculus and calyx can be traced back to four clusters of cell bodies. The γ lobe fibres however show no such symmetry. The staining of the developing structure suggests that the γ lobe is formed from relatively older cells whose positions were determined so long ago, or have been subsequently re-organised, such that they have lost any geographic identify they may have shared with their clonal partners (further evidence of this is provided in Chapter 5). The cell bodies that supply most the α and β lobes however, may be formed later and presumably, after any gross Kenyon cell re-organisation takes place. Thus the positions of the cell bodies of the α and β lobes still lie relatively close to their neuroblast precursors.

4.6 Summary

The staged developmental expression patterns described above would appear to confirm that much (but not all) of the larval mushroom body fibres degenerate and new ones form. The fibres that survive are most likely closely related to the γ lobe.

The α and β lobes are most likely new structures formed during pupation. It is possible that at least some of the cell bodies of the α and β lobes may be present in the larval structure, but their fibres are degenerated and regrown during metamorphosis. One of the P{GAL4} expression patterns displayed an obvious temporal change in expression. Line c772 did not stain in the larval or developing pupal mushroom body. The expression pattern suddenly appeared late in pupation, just prior to hatching. It may represent a gene only required for adult function in the mushroom bodies. The slow gradual change in the other expression patterns suggested such changes were structural. However, temporal changes in gene expression cannot be ruled out for any of these lines.

Chapter 5. HU Ablation of the Mushroom Bodies

5.1 Introduction

Hydroxyurea (HU) ablation of the proliferation quartet neuroblasts at the first larval instar effectively ablates the mushroom bodies (deBelle, J.S. and Heisenberg, M., 1993). This ablation was shown to result in the loss of olfactory associative learning capabilities. However, at the time of ablation, a small number of Kenyon cells already form the embryonic mushroom body (Ito, K. and Hotta, Y., 1992). Autofluorescence microscopy of ablated flies revealed no trace of the mbs in the brains of ablated adults. A small number of surviving fibres, forming a much reduced structure invisible to this histological technique, cannot be discounted. In the same study, 'partially' ablated individuals with reduced mb structures on one side were reported. Presumably, these partial ablations were due to a single neuroblast surviving the chemical treatment.

A number of P{GAL4} expression patterns were ablated using HU to generate both partial and completely ablated brains, and to examine, in detail, the effect of ablation on mushroom body substructure. A range of P{GAL4} lines was chosen that encompassed the anatomical subdivisions of the mbs described in Chapter 3. A selection of lines that stain extrinsic components of the mbs, and neurons of the antennal lobes were also examined.

Complete ablation was carried out in an attempt to investigate how many (if any) Kenyon cells survive from the embryo through to the adult, and to where their fibres project. Partial ablated preparations were generated in an attempt to investigate the nature of the four central tracts of the pedunculus, the four clusters of cell bodies and their relationships to the staining patterns observed in the lobes. Is each cell body

cluster derived from a single neuroblast? Do each of the four cell body clusters supply fibre bundles with identical projection patterns, or does each supply a specified region? HU ablation approaches are discussed in more detail in Chapter 2.

5.2 Effect of HU ablation on P{GAL4} expression in the adult mbs

5.2.1 Complete ablation of the mb expression patterns

A number of lines expressing in previously described subregions of the adult mushroom bodies were ablated using HU. In all cases, the surrounding neuropil, with the exception of the antennal lobes, appeared unaffected by the ablation of the mbs; the staining pattern looking identical to the non-ablated brain with the obvious exception of the missing mb expression pattern (fig 5.1). This confirmed that the HU treatment was only affecting neuroblasts that give rise to the antennal lobes and mushroom bodies. A few lines, all of which stain in the γ lobe of the adult, showed staining of a small group of Kenyon cells that were resistant to the ablation technique (fig 5.2). These surviving fibres all had medial projections into the lobes that resembled the projection patterns described for the γ lobes (cf. Appendix 1). Not all P{GAL4} lines that normally stain the adult γ lobes exhibited stained fibres after HU treatment. The number of surviving fibres in each case was very low, (less than 40) and even combined, would be insufficient to account for any major proportion of the normal γ lobe. As described previously, the γ lobe fibres project along the outer rim of the pedunculus. In the flies where a small portion of γ remained, there was no evidence of any ring-like structure in the pedunculus, suggesting that none of the internal structure of the pedunculus survives (i.e. no α or β fibres).

Fig 5.1 Complete HU ablation of mushroom body expression patterns

The figure shows the normal adult expression patterns (left) contrasted with the ablated brain expression patterns (right). Note that in all the lines, the only structures affected by the ablation are the mbs and the antennal lobes. (a) c739: The normal expression pattern of this line is predominantly restricted to the α and β lobes of the mbs, intrinsic interneurons of the antennal lobes and, neurons within the optic lobes and a group of transverse fibres (arrows). In the ablated fly, the mb and antennal lobe staining is lost completely. The staining of the transverse fibres and in the optic lobe is unaffected. (b) c492b: Normally stains a large number of Kenyon cells in all three lobes, extensively within intrinsic neurons of the antennal lobes and, a pair of very large posterior cell bodies (arrows), unclear in the reconstructions shown as the mb staining obscures them. Ablation of line c492b abolishes staining in both the antennal lobes and mbs leaving only the pair of large cell bodies that are clearly revealed (arrows) and appear to be the giant fibre descending neurons. (c) c35: This line stains a large amount of central brain neuropil but is strongest within the mushroom bodies where is shows staining in all three lobes. (right) No trace of mushroom body staining can be observed after ablation. All the surrounding neuropil, as far as can be determined, appears normal.

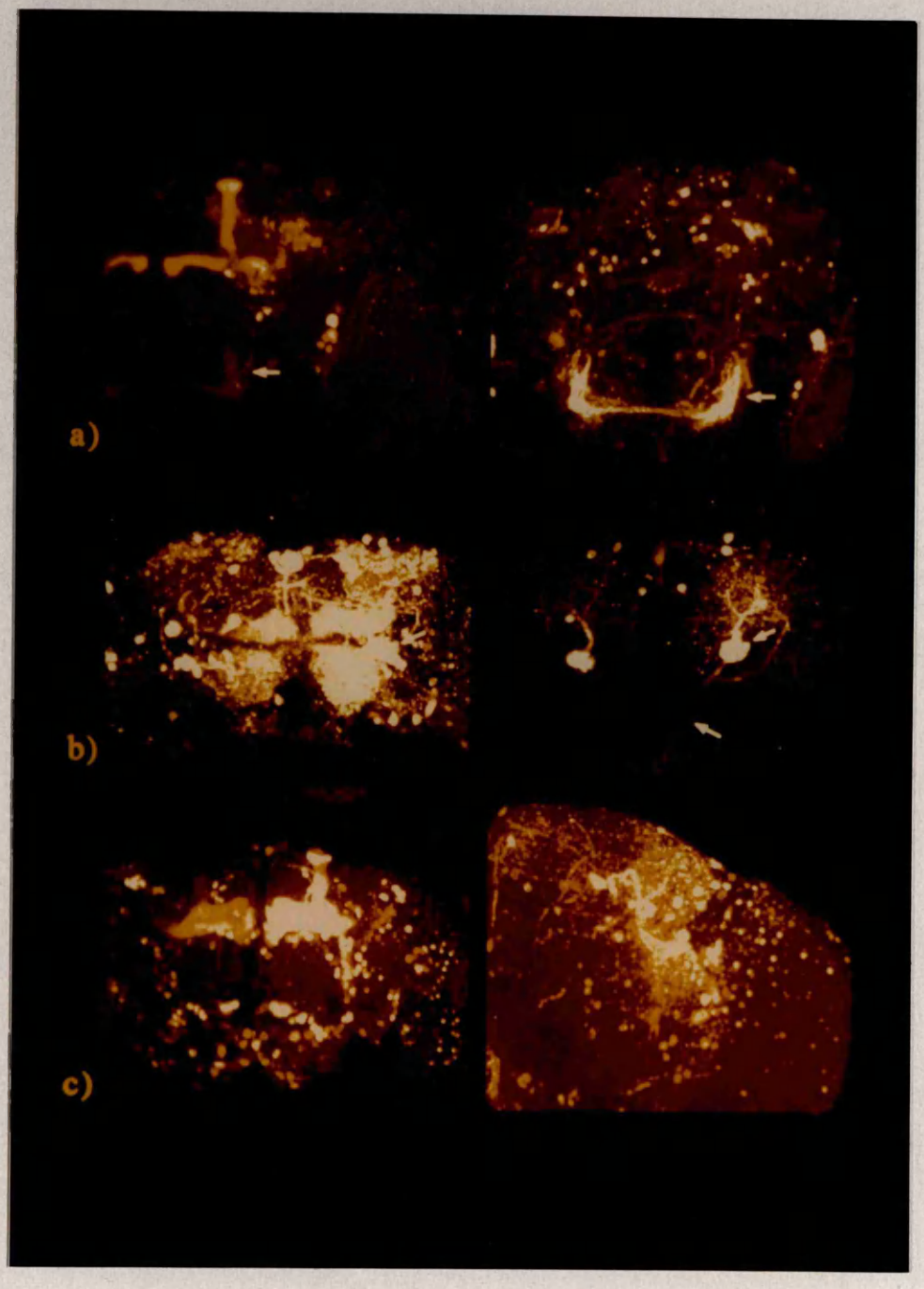
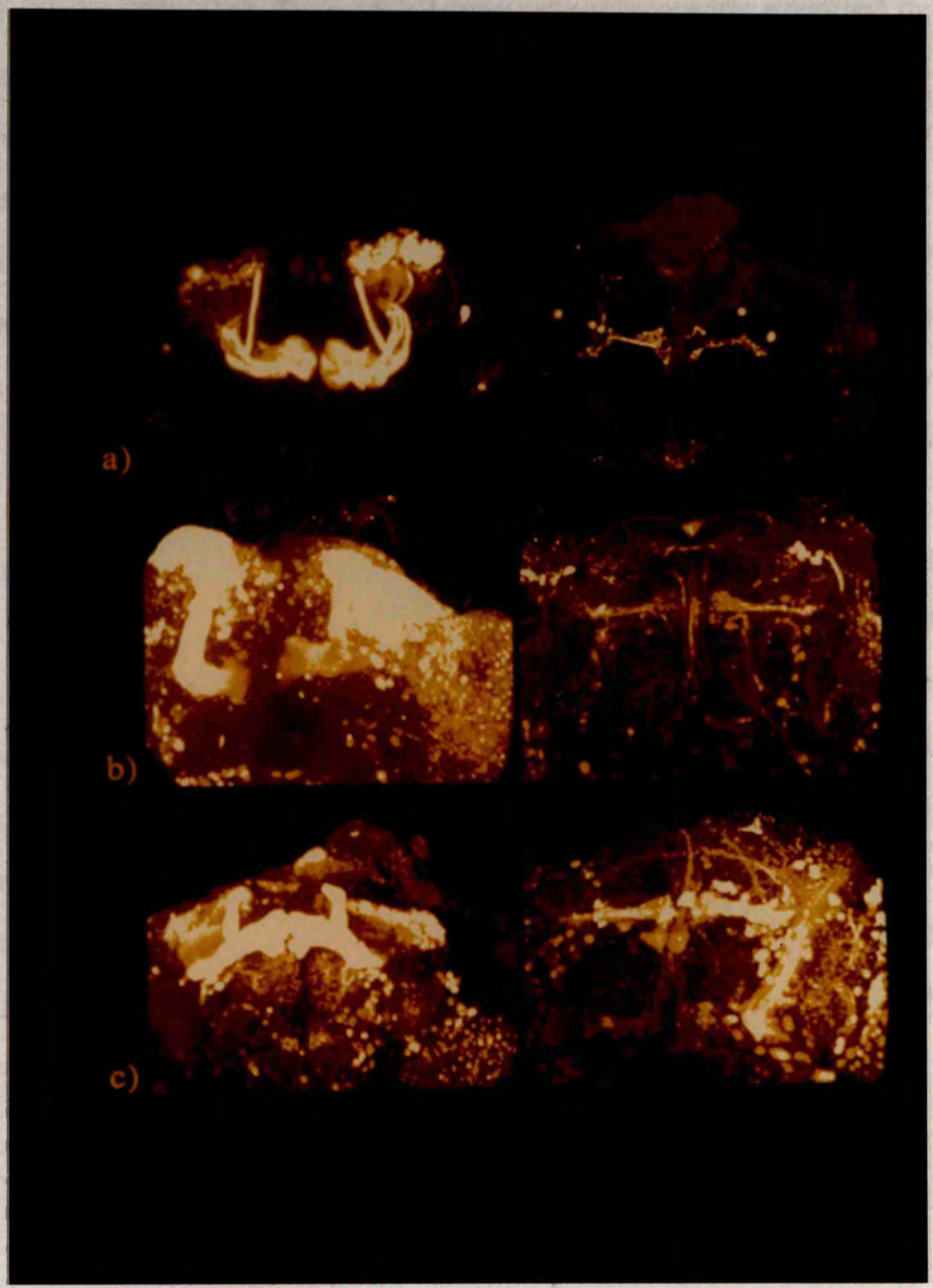

a)
b)
c)

Fig 5.2 Kenyon cells that survive HU ablation

The above figure illustrate a small population of Kenyon cells that always survive HU ablation. The 'normal' adult staining pattern is placed on the left of the figure to contrast with the pattern in the ablated individual on the right. (a) 201Y: The normal staining pattern in the adult brain shows staining of core regions in the α and β lobes, the γ lobes and of a small group of lateral cell bodies. After HU ablation of the proliferation quartet, only a small group (less than 10) Kenyon cells are stained in either hemisphere. These cells project in a fashion similar to that observed in the γ lobe of normal flies. (b) 30Y: Most neuropil regions of the brain stain in this line although, expression in the mbs is stronger. After ablation only a small number of Kenyon cells can be visualised again exhibiting a γ-like projection pattern. The surrounding neuropil appears unaffected by the HU treatment. (c) c772: Similar to 30Y, this line stains a large amount of central brain neuropil. In the mbs, the γ lobes and the outer regions of the α and β lobes are stained. After ablation, the non-mushroom body neuropil is unaffected. In the mbs however, only a few γ-like fibres stain.


a)
b)
c)

5.2.2 Partial Ablation of mushroom body expression patterns

A total of approximately three hundred individuals were treated with hydroxyurea at either 10mg/ml or 50mg/ml. Their adult brains were subsequently examined by either fluorescence or confocal microscopy (not all were scanned). The 10mg/ml concentration was used in an attempt to generate partial ablation of the mb neuroblasts. Only one partial ablation was isolated from the 100 or so individuals treated with HU at 10mg/ml. Six partially ablated brains were isolated from the 200 individuals treated at 50mg/ml. Six of the individuals that exhibited partial ablation stained a reduced mb structure in one brain hemisphere (asymmetric) (fig 5.3). Only a single individual showed evidence for a possible bi-lateral (symmetric) partial ablation (fig 5.4). In the lines where complete ablation left no trace of mb structure, the partially ablated structures resembled much thinner versions of the non-ablated patterns. The cell bodies were restricted to single, densely populated groups, and there was no evidence for subdivision within pedunculus. In lines where a group of γ lobe fibres are evident after ablation, the partially ablated structure shows an increase in the γ lobe staining and appearance of reduced α and β lobes. The cell bodies were again closely grouped and there was no symmetrical division of the pedunculus. All partially ablated mbs appeared to be no more than one quarter (by size) of the normal expression patterns, therefore, it would appear most probable that these patterns represent survival of just a single neuroblast

Fig 5.3 Asymmetric partial HU ablation of mushroom body expression patterns

3D Confocal reconstructions of the partially ablated mb structures. Each of the lines shows what appears to be the Kenyon cells produced from a single surviving neuroblast from the proliferation quartet. Compare with figures 5.1 and 5.2 for normal and complete ablation staining patterns. Note that in each case, the cell bodies are in a single, dense cluster. (a) Line 201Y shows staining of the α and β lobes in the partially ablated hemisphere. The staining in the γ lobe is also markedly increased. (b) Line c739, slim α and β lobes are present on only one hemisphere and completely absent on the other. (c) Line c772 shows staining in the α and β lobes in the partially ablated hemisphere. The volume of γ lobe staining is also increased in the partially ablated hemisphere.

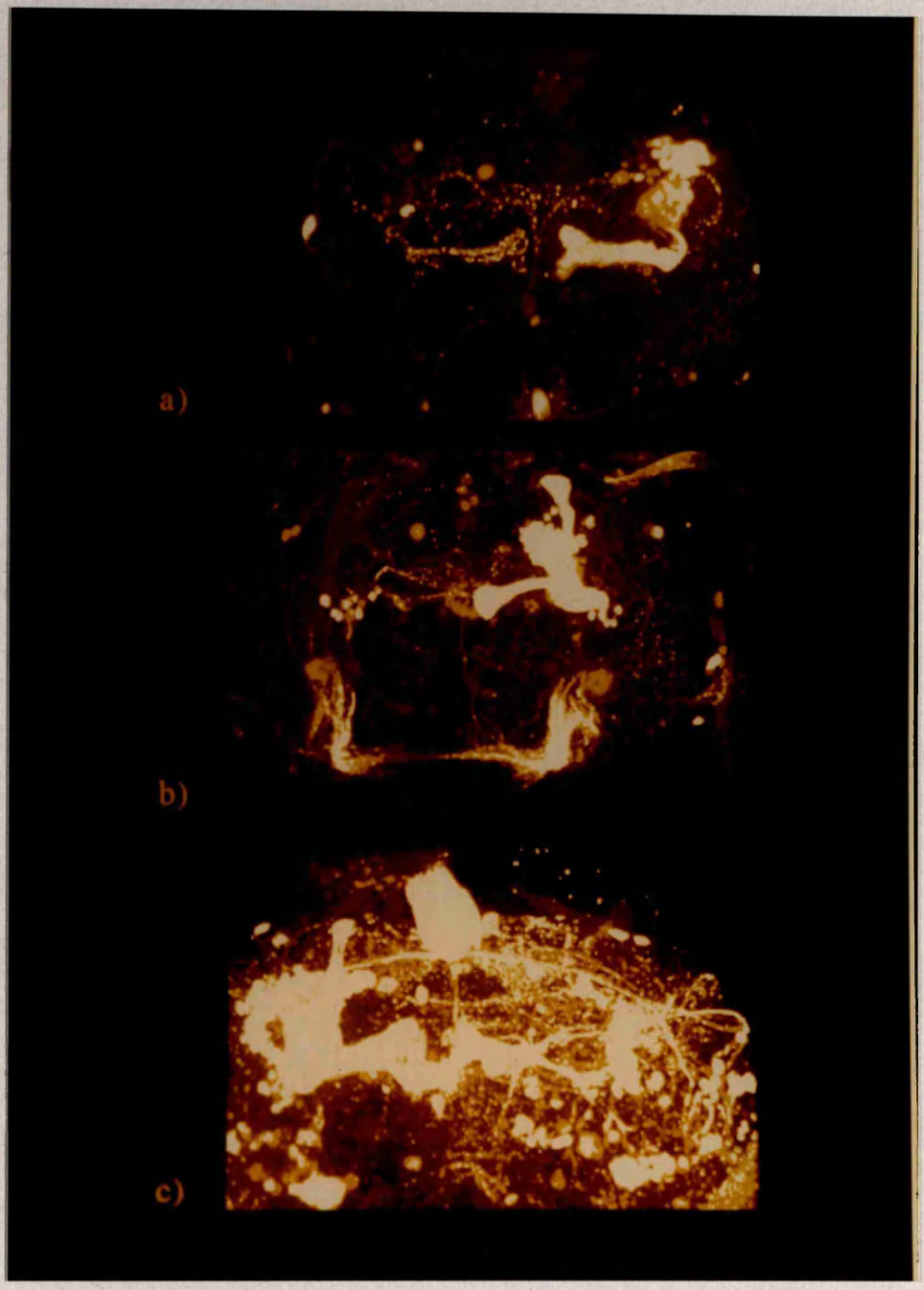
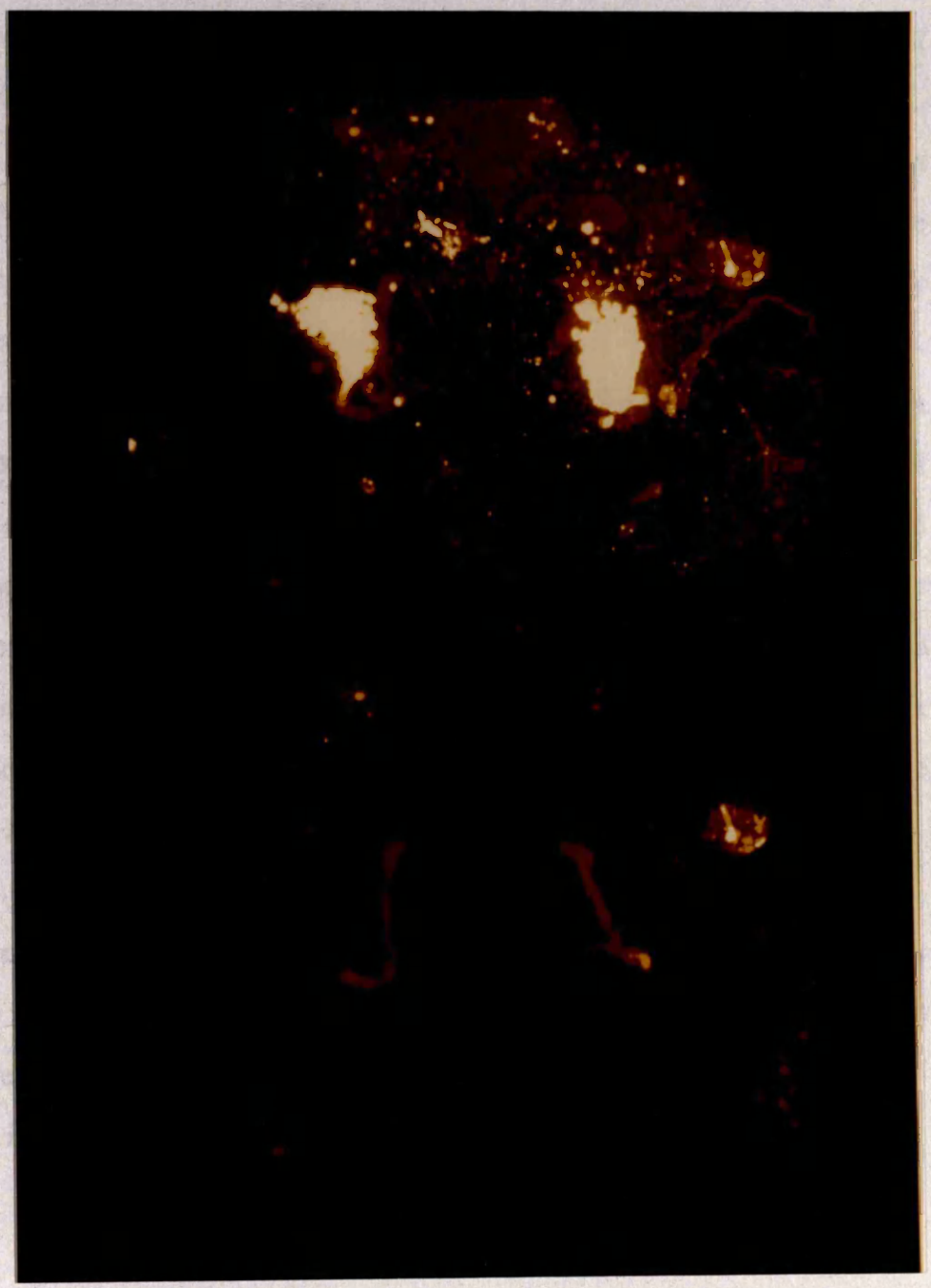

a)
b)
c)

Fig 5.4 Symmetric partial HU ablation of mushroom body expression pattern c739
In a single individual, reduced mushroom bodies were evident in both hemispheres after HU treatment. The upper panel shows a confocal reconstruction through region of the cell body layer and calyx. The lower panel shows a reconstruction at the level of the lobes.



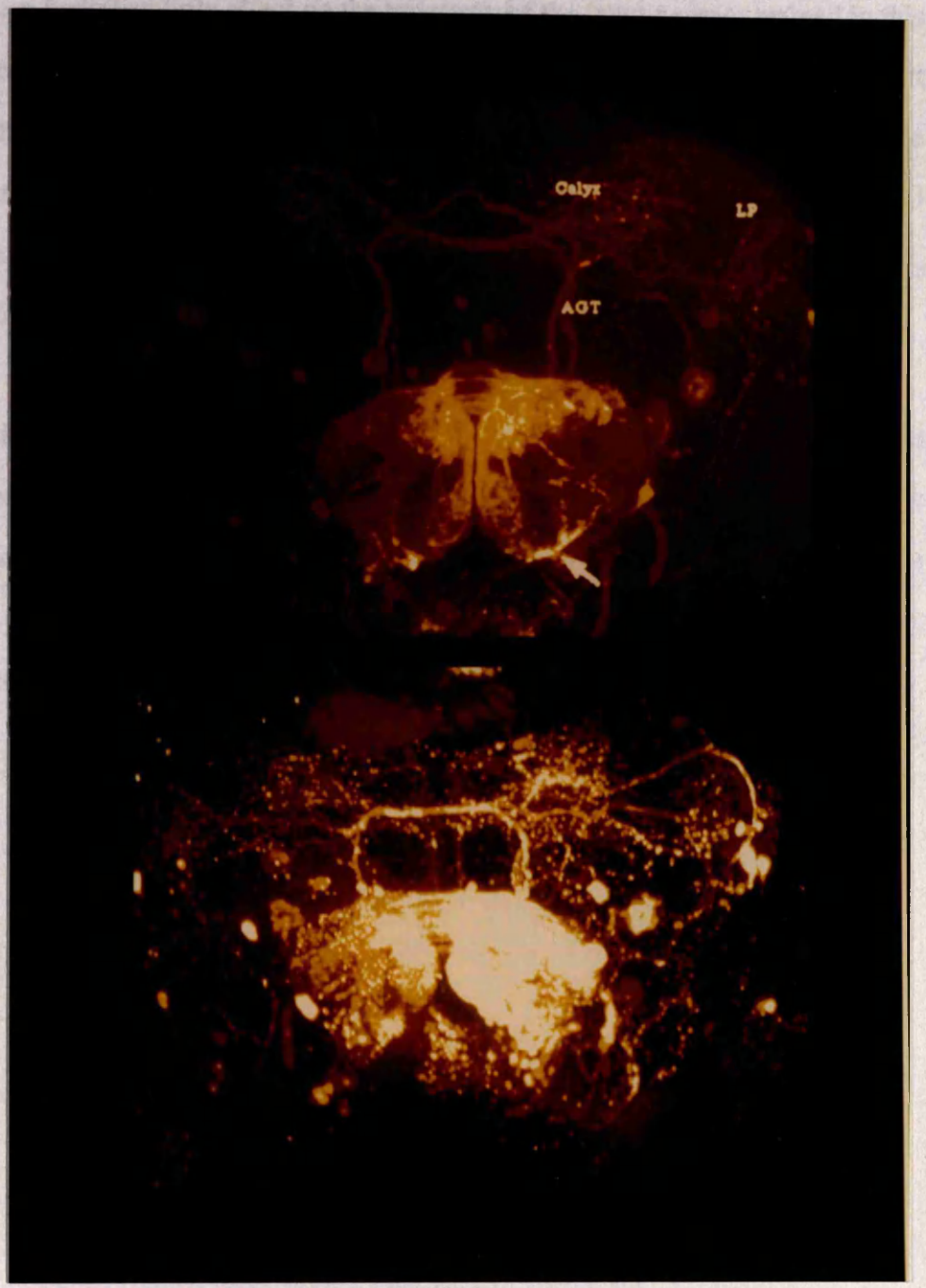
5.2.3 Ablation of the antennal lobes

In addition to the mb lines, a selection of antennal lobe staining lines were examined. The antennal lobes are large glomerular structures, found at the frontal margin of the brain ventral to the lobes of the mushroom bodies (Strausfeld, N.J., 1976). Like the mushroom bodies, they comprise intrinsic and extrinsic neurons (Stocker, R. F., et al., 1983, Stocker, R.F., et al., 1990, Stocker, R.F., 1994). The extrinsic neurons of the antennal lobes carry input from chemosensory and olfactory receptors around the body, the majority of which, enter through the antennal nerve. Their output is through the antenno-glomerular tract (AGT) which projects up to a region of arborisation within the calyces and then continues on to the lateral protocerebrum and lateral horn. The intrinsic neurons have their cell bodies located in a band across the frontal margin of the brain, just ventral to the γ lobes of the mushroom bodies. They provide local interneurons and generally form large diffuse clouds of processes within the antennal lobes structure. The lateral neuroblast that has an activity pattern similar to the proliferation quartet gives rise to antennal lobe neurons and HU ablation results in diminished antennal lobe size.

Several of the mb expression patterns also stain the antennal lobes, in particular, the intrinsic interneurons. Lines c739 and c492b are particularly good examples, though there are many others. This antennal staining is completely abolished after HU ablation in all the mb lines examined (figs 5.1).

Fig 5.5 HU ablation of antennal lobe line c133

The figure above illustrates the effects of HU ablation on extrinsic neurons of the antennal lobes. The normal pattern is shown above the ablated pattern. c133 stains a set of neurons that enter the antennal lobes through the antennal nerve (arrow). There, they arborise in a set of dorsal and medial glomeruli. There is also staining of a small set of neurons that leave the antennal lobes through the AGT and reach towards the calyces and lateral protocerebrum. The pattern after HU ablation is remarkably similar. The antennal lobes appear somewhat smaller but retain their glomerular structure. The output fibres of the AGT are also visible and appear to be unaffected.


Calyx
LP
AGT

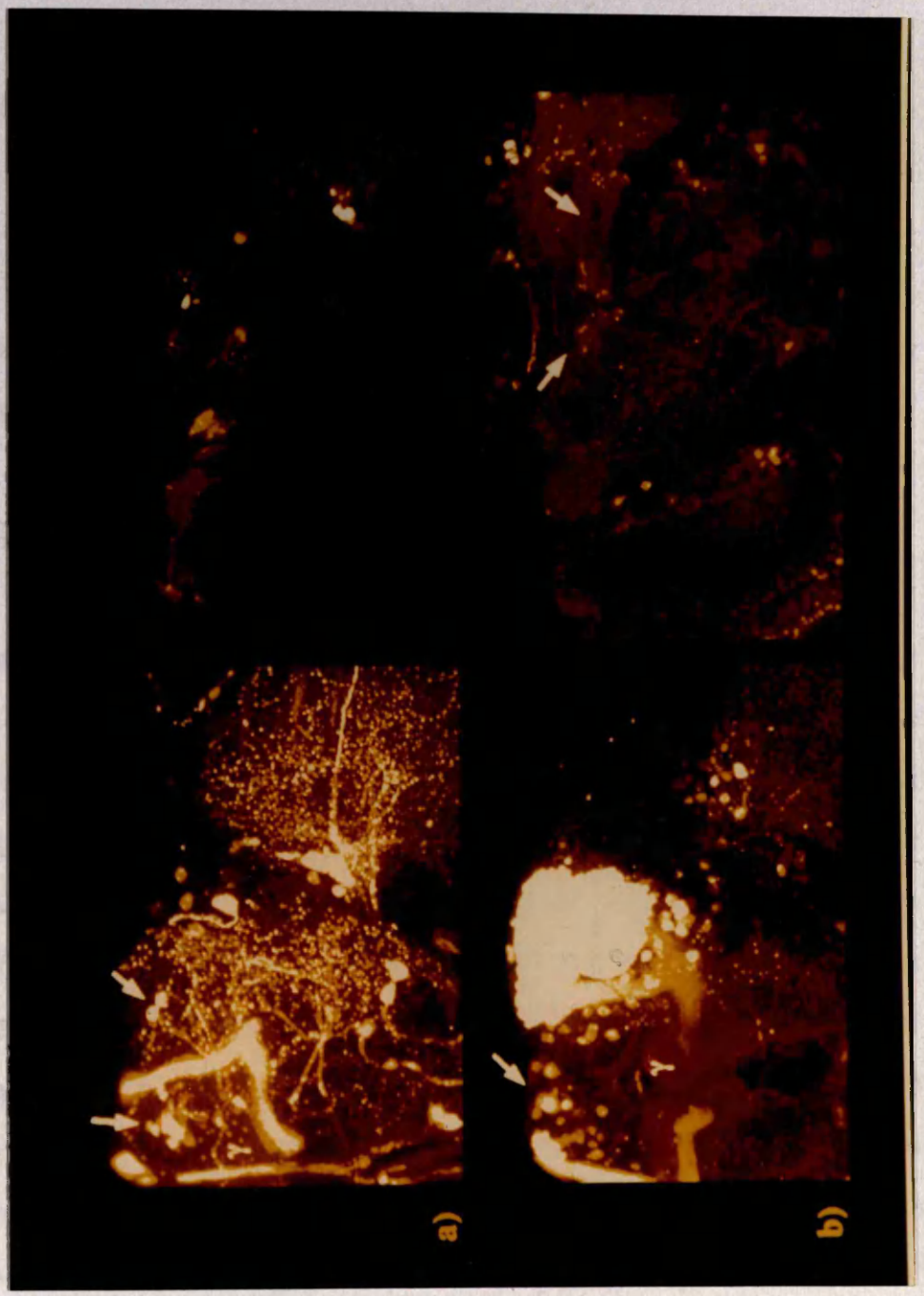
5.2.4 Ablation of mushroom body extrinsic neurons

HU ablation of line c133 (fig 5.5) clearly illustrates that HU ablation of the mushroom body has little effect on the trajectory of the input neurons stained in this line. The AGT fibres from the antennal lobes apparently find the calyx, despite it being severely reduced in size and fibre number. The effect of HU treatment on line c133 is restricted to a small reduction in the size of the antennal lobes. Previous HU ablation studies reported a similar reduction in size (deBelle, J.S. and Heisenberg, M., 1993).

In addition, two lines, 43Y and 238Y, stain populations of Kenyon cells and also a small set of mb extrinsic neurons, that appear to be γ lobe output fibres (fig 5.6). The projection pattern of the neurons that invade the γ lobe in these two lines is only slightly affected by HU ablation, the only visible effect being the reduced size of the arborisation pattern that coincides with the γ lobe.

Fig 5.6 HU ablation of γ output fibres from mb lines 43Y and 238Y

The lines above both stain Kenyon cell populations. In addition, both stain a small group of anterior dorsal cell bodies that send projections into the γ lobe. Photographs on the left reveal the normal γ lobes structure in these lines. To the right, are the ablated expression patterns in the same lines. (a) Line 43Y shows staining of Kenyon cells supplying the α and β lobes. The faint staining in γ is a result of a small group of extrinsic fibres. After ablation (left) , the intrinsic Kenyon cell staining is completely ablated leaving the γ output fibres alone. (b) Line 238Y stains Kenyon cells that send projections to all three lobes. Close examination of confocal sections reveals a small group of cell bodies similar to those in line 43Y. HU Ablation removes all the Kenyon cells staining leaving the extrinsic cells alone.


a)
b)

5.3 Discussion

5.3.1 Derivation of Kenyon cells from the proliferation quartet neuroblasts

At the developmental stage when HU is given to the larvae, the only active neuroblasts in each hemisphere are the proliferation quartet and the lateral neuroblast (fig 1.3). The selective ablation of most Kenyon cells and intrinsic antennal interneurons conforms to this pattern of neuroblast activity. Ablation at this developmental time confirms that mb development is dependant on the proliferation quartet neuroblasts.

5.3.2 Origin of surviving Kenyon cells

A few of the expression patterns reveal that a small number of Kenyon cells are resistant to HU ablation. Within any single line, staining of fibres that survive ablation is constant throughout a number of individuals, thus survival of these fibres is unlikely to be due to poor timing of HU treatment. Two possibilities for their origin exist. They are either formed in the embryo prior to HU treatment, or arise from different neuroblasts active at different stages of development. The latter case is unlikely as BrdU incorporation studies have been unable to identify any additional neuroblasts that give rise to Kenyon cells (Ito, K. and Hotta, Y., 1992). The surviving cells are thus most likely surviving remnants of the embryonic mushroom body. Previous studies have suggested between 40 and 300 Kenyon cells originate before larval hatching. In total, less than 100 Kenyon cells are stained in the ablated patterns when considered together. If the lines chosen represent all Kenyon cells then it would suggest that either the lower number of Kenyon cells formed in the embryo is more accurate, or that not all Kenyon cells formed in the embryo survive to the adult stages in the ablated brains.

5.3.3 Representation of embryonic Kenyon cells in the adult mb structure

Assuming the projection pattern of the Kenyon cells that survive HU treatment has not been affected by the treatment either directly, or indirectly through the lack of surrounding fibres, they resemble the projection pattern of fibres that go to the γ lobe alone. These fibres are only evident in lines that stain the γ lobe in the adult. If, as is most likely, these fibres are originating in the embryo, it provides further evidence that the γ lobe neurons are formed earlier than those of the α and β lobes. No evidence for the survival after HU ablation of any α or β lobe neurons was observed.

5.3.4 Clonal nature of the cell body clusters and structural four fold symmetry

In the adult brain and, at other developmental stages, a four fold symmetry is apparent in much of the mushroom body substructure. The most likely explanation is that each cell body cluster is derived from a single neuroblast. In addition, at any single stage in development, the cells derived from a single neuroblast probably have a similar projection pattern to the other Kenyon cells originating at the same time from the other three neuroblasts. An alternative is that each neuroblast provides Kenyon cells that derive specific substructure; i.e. one neuroblast could supply γ fibres alone etc. No symmetrical substructure is evident in partially ablated flies. In such individuals, there is a single cluster of cell bodies, a single tract in the pedunculus and, although reduced, a complete representation of the normal pattern in all of the lobes. Although most of the symmetrical divisions in the adult are related to α and β lobe Kenyon cells, partial ablation in lines that stain the γ lobes show that there is also a substantial element of γ formed from each neuroblast after larval hatching.

5.3.5 Targeting of mb extrinsic elements

Ablation of lines staining mb extrinsic fibres reveal that their projection patterns are more or less unaffected by HU ablation. The fibres that project to the lobes remain, although the area stained is reduced, reflecting the smaller size of the mb structure present in the ablated flies. Unfortunately, no lines staining α or β lobe extrinsic fibres had been isolated, thus the nature of these elements remain unknown. As γ lobe fibres survive from the earliest stages of mushroom body development also travel in the outer rim of the pedunculus, it is possible that these fibres have a structural role in determining the shape of the developing mushroom body, and may even provide targets for developing extrinsic elements. It is also possible that the mb extrinsic fibres are providing the target for the Kenyon cells.

5.3.6 Ablation of the antennal lobes

Many of the mushroom body staining patterns also stain intrinsic fibres of the antennal lobes. All lines isolated that stain intrinsic neurons of the antennal lobes show at least some degree of mushroom body staining. The lateral neuroblast also shows a similar activity pattern to that of the proliferation quartet. As only one neuroblast is present on either side, no partial ablation as described for the mbs is possible. In all cases, HU ablation leaves no trace of any antennal lobe intrinsic neurons. It is possible that all the intrinsic neurons of the antennal lobes arise from just the lateral neuroblasts. Those formed before larval hatching may not be stained by any of the lines isolated, or may not survive into the adult structure. Ablation of these neurons does not affect the projection patterns of the input neurons although the whole structure is reduced in size by approximately 30% (deBelle, J.S. and Heisenberg, M., 1993). Complete ablation of the local interneurons of the antennal lobes could certainly account for the previously described defects in olfaction.

5.4 Summary

HU ablation of the mushroom body neuroblasts at the early third instar stage effectively removes all trace of the mushroom bodies, with the exception of a small number of α lobe Kenyon cells. This supports suggestions that the adult γ lobe is related to the larval mushroom body and the α and β lobes are formed later in development. Partially ablated individuals, where a single neuroblast survives treatment, stain in a subset of all the normal structures, indicating that the four neuroblasts are most likely identical. At any particular stage in development, the neuroblasts would appear to be giving rise to Kenyon cells with similar projection patterns. Mushroom body extrinsic fibres, both input and output, appear relatively unaffected. HU treatment appears to ablate all of the antennal lobe intrinsic neurons in all lines examined. None of these antennal lobe intrinsic neurons were seen to survive into the adult.

Chapter 6. Discussion

6.1 Genetic dissection of mushroom body sub-structure using the enhancer-trap system

Since the patterns described here portray, for the most part, novel aspects of mushroom body structure, it is important to be clear as to what neuronal elements are represented. By the following criterion, the patterns are clearly due to GAL4 expression within Kenyon cells: staining in the lobes is invariably associated with staining in the pedunculus, the calyx, and with cell bodies in a cortical region above and behind the calyx. All descriptions of Kenyon cells, irrespective of insect species, refer to their origin from small cell bodies in such a region (Schürmann, F.W., 1987). Each Kenyon cell body provides a single neurite. Its dendritic branch contributes to the calyx, post-synaptic to afferents from olfactory and other sensory regions; its axon projects out of the calyx to form the pedunculus and lobes. Although flies were screened as P{GAL4} heterozygous, it is conceivable that the P-element insertion itself could cause dominant mutations with anatomical consequences (Cooley, L., et al., 1988). However, each of the lines has wild-type mushroom bodies as judged by interference phase contrast microscopy or autofluorescence. In other words, the projection patterns revealed by β-gal expression are contained within mushroom bodies of, at least approximately, normal size and shape. Different patterns therefore represent GAL4 expression in different, genetically specified, subsets of Kenyon cells.

Detailed analysis of enhancer-trap expression patterns has revealed a sub-structural organisation within adult *Drosophila* mushroom bodies, invisible to the techniques of conventional neuroanatomy. The active enhancers that drive reporter expression of the enhancer-trap are also presumably involved in regulating *Drosophila* genes

(Ghysen, A. and O'Kane, C., 1989). As many regulatory factors may be working on a single gene (or enhancer-trap), the expression pattern of the enhancer-trap element may not be identical to the expression pattern of any particular gene, yet the two expression patterns are related to some degree. The subdivisions with respect to enhancer-trap expression within the *Drosophila* mbs are therefore similar to those observed in the bee, where the calyx, pedunculus, and lobes are subdivided with respect to their expression of a number of neurotransmitters (Schäfer, S. and Bicker, G., 1986, Schäfer, S., et al., 1988).

Two main structural units became apparent in the above study. These structures, although previously identified, have never been described in detail for *Drosophila*. The mbs can almost be divided into the α/β lobe related structures and the γ lobe related structures. The former exhibit a high degree of concentric organisation at the level of the pedunculus and the lobes with a four fold symmetrical arrangement of cell body clusters, calyx subunits, and peduncular tracts. The γ lobe structures, although showing evidence of at least some substructure, are clearly not concentricaly subdivided and there is no evidence of cell body clustering or subdivision of either the calyx or pedunculus in any of the γ-lobe staining patterns.

Approximately 2200-3000 Kenyon cells are contained within a single *Drosophila* mushroom body, the exact number depending on age, sex, genetic background and environmental stimuli (Technau, G.M., 1984). Do the different patterns represent discrete or overlapping subsets of neurons? At least with respect to some lines, the patterns appear discrete. For example, the pattern of 201Y is in certain respects, the converse of the c739 pattern: In line 201Y, there is staining in the central components of α and β and in most of γ whereas, these areas are all unstained in line c739, which only stains an outer component of α and β, though there is probably some overlap at the core region of the α and β lobes. Some of the patterns examined, although not shown here, have outwardly identical staining patterns. If all thirty one

patterns were discrete, 2,200 cells would be insufficient to account for them. This raises the possibility that Kenyon cell identity may be specified by a combinatorial mechanism.

6.1.1 Genetic dissection of neuronal development using the enhancer-trap system

The difficulties in visualising identified neuronal structures and their components in the relatively stable adult brain are heightened considerably when applied to the developing brain. Of all brain structures, the mushroom bodies are one of those present at all developmental stages. Moreover, the gross structure of the embryonic mb is remarkably similar to that of the adult and all other stages in between (Ito, K., 1990). Cell body layer, calyx, pedunculus, and lobe system are easily identifiable in the mbs at all stages, yet, the mushroom bodies do change during development. These changes are more profound during metamorphosis when fibre number in the pedunculus drops sharply in the early stages, to rise again later (Technau, G.M. and Heisenberg, M., 1982).

The application of enhancer-traps to visualise brain structure in the adult brain has also been used here to visualise developing structure. The approach suffers from one major drawback. Gene expression may be temporally controlled, thus a gene expressed in one cell population at a particular stage in development may be expressed in different population at another stage (Bellen, H. J., et al., 1992a, Bellen, H. J., et al., 1992b). In an effort to overcome this, expression patterns were examined in a number of individuals at regular time intervals. A rapid change in expression pattern was observed in one line and was attributed to a change in enhancer activity rather than any gross structural change in the mbs. The rapid change in the expression pattern of line c772 during the latter period of pupation,

where many hundreds of Kenyon cells suddenly become visible late in pupation, cannot possibly be due to proliferation of new Kenyon cells, as such a growth rate would be too rapid (Ito, K. and Hotta, Y., 1992). All the other lines exhibited staining patterns that changed gradually over a much longer time period. Two other lines; c161 and c133, which in the adult, stain the central complex and antennal lobes respectively were also examined. These exhibited a similar, gradual change in staining pattern.

Although rapid gene expression changes may be discounted easily, slower changes can not. The staining of the γ lobe in the developing c739 pattern, which is lost before the adult stage, may represent such a slow change in gene expression. Environmental influences may also affect neuronal development (Heisenberg, M., et al., 1995). To control for this, all developmental analysis was carried out on larval and pupae reared under, as far as could be determined, identical conditions.

Previous studies have indicated a bundle of thin Kenyon cell axons in the centre region of the pedunculus (Technau, G.M. and Heisenberg, M., 1982). A structural role, in guiding the developing fibres during metamorphosis, was proposed for these fibres and it was suggested that of the larval fibres, these were the ones that survived into the adult mushroom body. These propositions were based on continuity of the axonal morphology of these axons in cross section alone. The developmental patterns described here suggest otherwise. All continuous staining of mb substructure has been linked closely with the γ lobes in the adult and the outer regions of the pedunculus. HU ablation of the mb neuroblasts leaves a remnant that again, is related to the adult γ lobe and outer regions of the pedunculus, rather than an inner core. During pupation, the formation of the adult α and β lobe structures appear to start with the outer most regions. The inner structure forms later, suggesting that any centrally located bundle of fibres are newly formed growing

fibres, and this may explain the differences in morphology between them and the older fibres surrounding them.

The gross structure of the mushroom bodies at different stages has previously been described as being similar at each developmental stage (Ito, K., 1990). In this study, it has been revealed that between the adult and larval forms, the characteristic appearance of the mushroom bodies (the lobe structure) whilst appearing outwardly similar, are quite distinct. The larval fibres appear to be more closely related to the γ lobe structures in the adult. The adult α and β lobe structures are formed from new fibres from either new cell bodies or, are possibly regrown from older cell bodies, whose fibres degenerated during early metamorphosis. Clearly, some of the fibres in the mushroom bodies do degenerate. The exact fate of the cell bodies whose fibres degenerate is unclear, though they probably regrow fibres (Technau, G.M. and Heisenberg, M., 1982, Ito, K., 1990) that project to the lobes. The Kenyon cells that project fibres to the α and β lobes lie in four small clusters, indicating that of the whole Kenyon cell population, these are the closest to the four neuroblasts and are thus, the last to be formed (Ito, K. and Hotta, Y., 1992). It is most likely that at some stage during development, the four proliferation neuroblasts switch from production of Kenyon cells that project fibres in to the α lobe, and start producing the α and β lobe related neurons. Thus, it is most likely that any regrown fibres would project to the γ lobe structures.

6.2 From structural to functional subdivision

6.2.1 Multiple computational channels within the mushroom bodies

What role, if any, does the novel sub-structure of the mushroom bodies described here play in the function of the neuropil? The mbs have been implicated in a wide variety of behavioural responses in insects. In *Drosophila*, roles in processing olfactory information and in courtship behaviour have been suggested (Davis, R.L.,

1993). The patterns of reporter expression in the enhancer-trap lines described here imply that Kenyon cells in the mbs can be subdivided with respect to their gene expression. Cellular heterogeneity in terms of gene expression suggests that the mbs are multiplex neuropils, in which different components may serve different functions. Such observations are not without precedent. Golgi impregnations of the bee calyx reveal subdivision by dendrite morphology and zoning of afferents (Mobbs, P.G., 1981) while bee lobes show subdivision in terms of transmitter expression (Schäfer, S. and Bicker, G., 1986, Schäfer, S., et al., 1988). In addition, certain areas of the cockroach mushroom body may mediate different integrative functions, such as motor planning and place memory (Mizunami, M., et al., 1993). However, the extent of subdivision revealed by the enhancer-trap method, previously invisible to the techniques of classical neuroanatomy, is remarkable.

Whether these subdivisions represent parallel subdivision in functionality is unclear. The α and β structures in the adult, with their concentric organisation, lend themselves readily to such classification and may well function in this manner. The concentric divisions of the α and β lobes in *Drosophila* can be thought of as very similar to the parallel division observed in the bee lobes, revealed by immunoreactivity against neurotransmitters. The different regions in the α and β lobes may be responsible for processing different groups of olfactory stimuli. The γ structure shows different structural characteristics to the α and β structures, such as a diffusely distributed cell body layer and a single diffuse calyx, and shows no such concentric subdivision of either the pedunculus or lobe. The γ structure may therefore be quite functionally distinct from the α and β lobes.

Portions of the γ lobes appear to survive from the larval brain into the adult. *Drosophila* have been reported to retain memory through metamorphosis (Cambiazo, V., et al., 1994). Conditioned odour avoidance responses produced in larvae have were shown to be present in adults after a period of eight days. If, as is

believed, olfactory associated memory is situated at synapses (Hawkins, R.D., et al., 1993) within the mushroom bodies (Davis, R.L., 1993), it would suggest that at least some of these survive pupation. Thus, if larval memory does survive in the mushroom bodies, it most likely reside in the γ lobes. As some of the γ lobe structure is resistant to HU ablation at the early first instar larval stage, it may also be responsible for the low level of olfactory associative learning displayed by ablated flies (deBelle, J.S. and Heisenberg, M., 1993).

The development of functionally related brain structures at specific developmental stages may in some cases be linked. For example, the central complex involved in higher order visual processing forms late in pupation as the development of the optic lobe is finishing (Ito, K., 1990). While the antennae and antennal lobes develop to form the adult olfactory system, the α and β lobes of the mbs also form. It may be that these lobes are involved in processing signals from the adult olfactory system, and the older γ lobe related structures in the adult are involved in processing more basic chemosensory cues common with the larval environment. The α lobe also changes during metamorphosis, and this may reflect functional modifications between the larval and adult structures. It may be that all memory functions of the mushroom bodies are located within the α lobe, with the adult α and α lobes involved in other aspects of behaviour. The larval fibre degeneration may represent the functional redundancy of these fibres within the adult environment.

The strongest evidence for functional subdivision comes from another application of the P{GAL4} enhancer-trap. GAL4 was used in this study to drive expression of *lacZ* but may be used to drive practically any gene placed downstream of the UAS_G recognition site. Described in Appendix 2, is a study where *tra*, a major gene in the sex determination pathway, was expressed in mushroom body staining enhancer-trap lines. In *Drosophila*, sex determination is cell autonomous in that the sex of a cell is determined by genetic factors within the cell and not by external factors. *tra* has two

transcripts, one male which is inactive, and one female, which is active and dominant (Boggs, R.T., et al., 1987). Expression of the female *tra* transcript within a cell effectively feminises the cell (McKeown, M., et al., 1988). The female transcript of *tra* may be expressed under GAL4 control (Ferveur, J.-F., et al., 1995). Selective feminisation of mushroom body subunits by GAL4 mediated *tra* expression provides the strongest evidence for functional subdivision in the mushroom bodies. Expression of the feminising factor, *tra*, in different regions of the mushroom bodies in male brains has different effects on the courtship behaviour of the flies. In some lines, courtship behaviour is unaffected whilst in others, the males start courting other males as well as females. Precisely which mb subcomponent is involved in this behavioural transformation is, as yet, unclear, as insufficient lines have been tested.

6.2.2 The olfactory system as a single higher order computational unit

In a complex field such as the study of behaviour, it is desirable to eliminate as many variables as is possible, in order to achieve a clear conclusion. By virtue of the direct connections between the two structures, the antennal lobes and mushroom bodies are often considered together by anatomists studying the olfactory pathways (Erber, J., et al., 1987, Davis, R.L., 1993, Menzel, R., et al., 1994, Strausfeld, N. J., et al., 1994). Studies of behaviour tend to focus on one or the other structure in isolation (Rodrigues, V. and Buchner, E., 1984, Ballinger, D. G. and Benzer, S., 1989, Han, P.-L., et al., 1992). In particular, in olfactory associative learning tests, if a test animal shows a response to a particular odour, it is generally assumed that the antennal lobes are functioning and any defects are usually attributed to defects at the level of the mushroom bodies. A particularly good example of this is the HU ablation tests of deBelle and Heisenberg (deBelle, J.S. and Heisenberg, M., 1993). After ablation, the test animals can not recognise one of the common noxious odours used in olfactory associated learning and memory tests, thus another test odour had to be substituted. The antennal lobes were clearly shown to be defective with respect

to at least one odour. Once two suitable test odours were chosen, defects in associating the odours with negative reinforcement were ascribed to the mushroom bodies.

In terms of both development and gene expression, the two structures begin to appear even more closely related that previously assumed. Many of the lines which stain in the mushroom body α and β lobes, also stain intrinsic neurons of the antennal lobes. In all lines examined, HU ablation resulted in the loss of staining of antennal lobe intrinsic neurons. Antennal lobe extrinsic neurons and the shape of the structure and glomeruli appeared unaffected by the treatment, though overall size was reduced. Apart from being unable to recognise certain odours, the effect of ablation on the intrinsic neurons of the antennal lobes is unknown. They may play an important role in association of multiple signals and in communication with the mbs.

The antennal lobes are certainly subdivided with respect to gene expression patterns, as revealed by immunoreactivity to a variety of brain specific antibodies (Störtkuhl, K.F., et al., 1994). As to whether the antennal lobes show the concentric organisation related to those of the mushroom body α and β lobes has not been investigated in any detail. A large proportion of the mushroom body lines also stain the intrinsic neurons of the antennal lobes. The antennal lobes are the most common secondary structure stained in the mushroom body lines, with the exception of the neuronal secretory cells of the par intercerebralis, which stains in almost all P{GAL4} lines. Both the antennal lobes and the mbs are involved in olfactory processing (Laurent, G. and Davidowitz, H., 1994, Stocker, R.F., 1994) and may be expressing similar genes. The degree of homology between the enhancer activity in both structures suggests the functional relationship between them may be much closer than previously described. It may be inappropriate to address any functional aspect of the α and β lobe structures without careful consideration of these antennal lobe intrinsic neurons. No significant relationship between the γ lobe Kenyon cells

and neurons of the antennal lobes or, for that matter, any other brain structure was observed.

6.3 Further work

In a recent review on the genetic approach to learning and memory (mnemogenetics) it was stated that 'The goldrush in *Drosophila* mnemogenetics is over' (Heisenberg, M., 1989). That same year, the first application of enhancer-traps to describe neuronal elements was published (Ghysen, A. and O'Kane, C., 1989). With the development of the P{GAL4} enhancer-trap and its use as a targeted expression system (Kaiser, K., 1993), it has become clear that the goldrush has not yet started.

This study has isolated a number of genetically defined structural subdivisions in the mushroom bodies, the region of the insect brain most closely associated with olfactory associated learning and memory. It has also revealed a complex and intriguing developmental pattern that links the mushroom bodies closely with the its lower order relative; the antennal lobes. The P{GAL4} system also provides powerful tools that may be used for further investigation, both structural and functional. Clearly, there is great scope for continuation.

6.3.1 Analysis of anatomical mutations

An obvious extension to this study would be morphology of the structural mutants in high detail. Analysis of the expression patterns within the mutant genetic background would hopefully reveal the nature of the disruptions with respect to the different subdivisions of the mushroom bodies throughout development (Heisenberg, M., 1980). The role, if any, of the outer γ fibres in providing a structural framework for mushroom body development may be revealed. HU ablation of the mushroom bodies also results in an overall reduction in the size of the

antennal lobes. The intrinsic component neuronal of the antennal lobes is small, thus any mutation affecting these cells may not produce measurable differences in the overall structure, as visualised by traditional anatomical techniques. As the intrinsic neurons of the mbs appear to be closely related in terms of gene expression to the antennal lobe interneurons, it is likely that such structural mutations that affect Kenyon cells may also affect the antennal lobe neurons in ways invisible to classical neuroanatomical approaches.

6.3.2 HU ablation and other developmental stages

According to the calculation of Kei Ito, based upon the fastest observed rate of division for neuroblasts, the four neuroblasts of the proliferation quartet are insufficient to account for the 2200-3000 reported Kenyon cells. Using the same calculations, the estimated number of Kenyon cells originating in the embryo is between 40 and 300 (Ito, K. and Hotta, Y., 1992). Only a few fibres (max. 40) have been stained in the lines described above. Staining of the embryo and HU ablation of the proliferation quartet, followed by analysis at other developmental stages, in particular, in the larval brain, would (hopefully) reveal whether there are any additional fibres in the embryo that are subsequently lost during pupation.

There are a number of other developmental stages where feeding HU is not actually lethal to the developing fly (Steve deBelle, pers comm). Whilst this will ablate other active neuroblasts, thus affecting other brain neuropil, it may resolve the exact origin of the fibres in the α and β lobes. If such fibres are formed from new cell bodies rather than regrown from ones which previously projected fibres into the larval structure, then ablation at later stages should first affect the α and β lobes which would only survive rather later ablation.

The interpretation of the developmental studies discussed here rely on an assumption that the patterns represent structural changes in fibre projection patterns during development rather than temporal changes in gene expression within different Kenyon cells. The only way to address this is by studying and cross-correlating a number of expression patterns and taking any other available data such as HU ablation at a variety of stages.

Several techniques are available which allow cell lineages to be traced. Incorporation of the labelled nucleotide 5-bromodioxyuridine (BrdU) into dividing neuroblasts in *Drosophila* has been used extensively (Truman, J.W. and Bate, M., 1988, Prokop, A. and Technau, G.M., 1991). Double labelling of staged pulses of BrdU with the expression patterns would allow confirmation of the relative age of the stained cells. The cell bodies of Kenyon cells that project to the γ lobe would only label if the pulse was early in development. Later pulses would only label the four clusters of Kenyon cells that send fibre projections to the α and β lobes.

6.3.3 Characterisation of the antennal lobes and other brain regions

Clearly, the relationship between the antennal lobes and the mushroom bodies is a strong one both in terms of their functional roles and gene expression patterns. Detailed characterisation of the antennal lobes, both in the adult brain and developmentally, using a number of P{GAL4} lines, may reveal substructural similarities. There may be several populations of intrinsic antennal lobe interneurons with projections to different glomeruli or, different regions within glomeruli. Application of this technique to other brain regions may reveal similar substructural subdivision invisible to the traditional techniques of neuroanatomy. The technique described here is not just specific to neurons: other tissues may well prove to be subdivided in terms of gene expression.

6.3.4 Single cell projection patterns

The quantity of information that can be extracted from a single P{GAL4} expression pattern is inversely proportional to the number of neurons it stains. Lines such as 201Y and 259Y, which stain relatively few Kenyon cells, provide a wealth of detailed information and allow some deduction of single neuronal projection patterns to be made. However, these patterns are rare and of all the lines stained, only 259Y stains less than 100 Kenyon cells. In some lines, the expression patterns are even more restricted. Line c561, for example, stains 6 neurons of the ellipsoid body in either hemisphere. Even in this staining pattern, single cell projections are difficult to resolve. A number of approaches may resolve this issue. The most obvious is to inject a single cell within a population with a dye. This approach has been investigated but to date, has been unsuccessful.

6.3.5 Functional characterisation of mb subunits

As discussed earlier, the P{GAL4} system allows expression of a range of constructs that may modify the properties of the cell that they are expressed in. The first such study using the P{GAL4} lines described here has indicated functional subdivision with respect to courtship behaviour in *Drosophila*. This is just one example of the type of constructs and behavioural tests that could be applied. Cell toxins, modifiers, transmitters, gene products to partially rescue phenotypes in null backgrounds, even antisense transcripts to knock out genes are all quite feasible using the P{GAL4} system (Brand, A. and Perrimon, N., 1993). Initial experiments using a temperature sensitive ricin A chain have been used to ablate mushroom body neurons (Moffat, K.G., et al., 1992).

Functional subdivision of bee mbs has been proposed due to its parallel subdivision in terms of gene expression. Analysis of transmitter immunohistochemistry at high

resolution within the *Drosophila* mushroom bodies would be desirable. The immunohistochemistry of the *dunce* protein within the mushroom bodies revealed elevated levels with respect to other neuropil (Nighorn, A., et al., 1991). Although the stained section in the original is not of a high resolution, it would appear that the staining pattern within the mb is not complete and that *dunce's* expression may be restricted to specific components of the mbs.

6.3.6 Environmental effect on mushroom body subunits

The fibre number in the pedunculus and total volume of the mushroom bodies have been shown to be influenced by a number of environmental conditions (see introduction). Such increases may be general but, if mb subunits are related to specific stimuli, the number of fibres encompassed by a single expression pattern may alter. The difficulty here is in measuring the effect of environmental conditions on specific expression patterns. Only a very large increase in any one sub region of the mb structure would be visible by confocal microscopy of fluorescent immunohistochemically stained whole brains. To quantitate such changes accurately, the fibre numbers would have to be counted under EM, using some of the EM dense immunohistochemical approaches. Although not discussed in the text, a number of stains were used for *lacZ* under EM, but none worked satisfactorily. A UAS_G reporter based on a peptide suitable for immunogold EM labelling may prove more suitable (Elekes, K. and Ude, J., 1993).

6.4 Conclusions

The application of the P{GAL4} enhancer-trap to investigate neuronal structure has proved worthwhile. It provides an additional tool for visualising neuronal structure in the adult and throughout it's development. Moreover, the P{GAL4} system then provides a mechanism for manipulating the neurons *in situ*. It has revealed structural

subdivision within the mbs, implying a similar functional subdivision. Preliminary findings based on expression of *tra* within mushroom body subunits also suggest a degree of functional subdivision within the structure. The findings described here answer few questions in themselves, but the approach used provides a possible means to test the hypotheses presented at a future date.

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