Development 124, 761-771 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV8383

The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells

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SUMMARY

The mushroom body (MB) is an important centre for higher order sensory integration and learning in insects. To analyse the development and organisation of the MB neuropile in *Drosophila*, we performed cell lineage analysis in the adult brain with a new technique that combines the Flippase (flp)/FRT system and the GAL4/UAS system. We showed that the four mushroom body neuroblasts (MBNbs) give birth exclusively to the neurones and glial cells of the MB, and that each of the four MBNb clones contributes to the entire MB structure. The expression patterns of 19 GAL4 enhancer-trap strains that mark various subsets of MB cells revealed overlapping cell types in all four of the MBNb lineages. Partial ablation of MBNbs using hydroxyurea showed that each of the four neuroblasts autonomously generates the entire repertoire of the known MB substructures.

Key words: *Drosophila*, brain, mushroom body, development, cell lineage, flp/FRT, GAL4/UAS, enhancer trap, hydroxyurea

INTRODUCTION

The brain consists of discrete regions that are specialised for unique functions such as sensori-motor coordination and learning. In vertebrates, the hippocampus is an important centre for learning and memory. Removal or local injury of this region impairs formation of a certain class of memory with minimal effects on other brain functions (reviewed by Kupfermann, 1991). In insects, the mushroom body (MB) plays a crucial role in higher order sensory integration and learning (Davis, 1993; de Belle, 1995; Davis and Han, 1996). Local cooling of the MB region disturbs the olfactory memory of honey bees (Erber et al., 1980). *Drosophila* mutants such as *mushroom bodies deranged* and *mushroom bodies miniature* have structural defects in this neuropile region that are accompanied by a learning deficit (Heisenberg, 1980; Heisenberg et al., 1985). Flies with chemically ablated MBs show severe defects in associative odor learning (de Belle and Heisenberg, 1994). Mutations in the *dunce*, *rutabaga* and *DCO* genes, which are involved in the cAMP signalling system, also influence olfactory learning. These genes are expressed preferentially in the MB (Nighorn et al., 1991; Han et al., 1992; Skoulakis et al., 1993; Davis et al., 1995). The MB is also involved in courtship behaviour. When certain parts of the MB are genetically feminised, the male flies cannot discriminate between the two sexes when looking for their mates (Ferveur et al., 1995; O'Dell et al., 1995).

Consistent with its role in such associative functions, the MB shows a high degree of structural plasticity. The volume and fibre number of the MB vary according to the age, sex and experience of individuals in *Drosophila* (Technau, 1984; Balling et al., 1987; Heisenberg et al., 1995) as well as in honey bees (Durst et al., 1994). This suggests that the generation and/or maintenance of the MB structure is under both genetic and epigenetic control.

The *Drosophila* MB is a paired neuropile structure. Each neuropile consists of about 2500 neurones called Kenyon cells (Kenyon, 1896; Hinke, 1961). The cell bodies lie in the dorsoposterior cortex of the protocerebrum sending fibres inwards to form the calyx neuropile, where they receive signals from the antennal lobe interneurones and from other interneurones of various sensory modalities (Power, 1943; Strausfeld, 1976; Stocker et al., 1990). From the calyx, the fibres extend anteriorly via the pedunculus to reach the anterior protocerebrum, where they form the α, α′, β and γ lobes.

In a previous study, we found that among the 85 neuroblasts in the larval central brain, four specific neuroblasts give birth to neurones in the MB cortex above the calyx (Ito and Hotta, 1992). These neuroblasts, called mushroom body neuroblasts (MBNbs), are four of the five neuroblasts that are mitotically active at the time of larval hatching (Truman and Bate, 1988; Ito and Hotta, 1992). This finding led to the development of a chemical ablation technique for MB cells by administration of the DNA-synthesis inhibitor hydroxyurea (HU) to newly

hatched larvae (Prokop and Technau, 1994). The technique is now widely used to assess the role of the MB in various behavioural assays (de Belle and Heisenberg, 1994).

Since the previous work (Ito and Hotta, 1992) utilised BrdU, which labels only nuclei, it was not possible to show whether the projections from the MBNb progeny are confined to the MB neuropile. In addition, the importance of the MB in higher-order brain functions and the abundance of Kenyon cells suggest that there are elaborate networks of highly differentiated neurones in the MB, as shown with enhancer-trap strains that mark various substructures within the MB structure (Yang et al., 1995). Thus, two important questions are left unanswered: (1) Do the four MBNbs exclusively generate Kenyon cells, or do they also generate other types of cells? and (2) What is the relationship between the MB substructures and the lineage of the four MBNbs? Are the four MBNb clones identical or different in terms of their cellular composition and function?

To answer these questions, we performed clonal analysis of the adult MB cells with a newly developed method that uses the yeast Flippase (flp)/FRT system in conjunction with the GAL4/UAS system. We present direct evidence that the progeny of the MBNbs contribute exclusively to the MB. It is likely that the MBNbs give birth not only to the Kenyon cells but also to glial cells. We also found that all four clones are structurally indistinguishable. This was further confirmed by comparing 19 GAL4 enhancer-trap strains, all of which labelled certain subsets of cells in each of the four clonal clusters. The partial ablation of the MBNbs showed that a single MBNb is sufficient to autonomously generate all the MB substructures labelled in these strains. Thus, we conclude that the MB is a fourfold structure of clonal units, each of which contains an essentially identical set of neurones and glial cells.

MATERIALS AND METHODS

DNA construct

The AyGAL4 construct uses the P-element transformation vector CaSpeR (Thummel and Pirrotta, 1991), which contains the following components in the 5′ to 3′ order (Fig. 2): Act5C promoter (Struhl and Basler, 1993), two hsp70 termination signals flanking the *yellow*$^{+}$ gene (Struhl and Basler, 1993), the *gal4* gene encoding a yeast transcriptional activator (Brand and Perrimon, 1993) and the SV40 polyadenylation signal. Germline transformation was done using w^{1118} as the host strain and pπ25.7wc (Karess and Rubin, 1984) as a helper plasmid. Detailed protocols of the plasmid construction are available upon request.

Clonal analysis in the larval and adult brain

Female flies homozygous for hsp70-*flp* (Golic and Lindquist, 1989; insertion in the X chromosome) were crossed to males homozygous for both AyGAL4 (insertion in the second chromosome) and UAS-*tau* (K. Ito, S. Schneuwly et al., unpublished, insertion in the third chromosome). The construct UAS-*tau* has a bovine *tau* cDNA under the control of the yeast upstream activation sequence (UAS).

To minimise spontaneous recombination caused by heat-shock-independent *flp* expression, the flies were kept at 16˚C, except when heat-shocked. Newly hatched F_1 larvae, hemi- or heterozygous for hsp70-*flp* and heterozygous for AyGAL4 and UAS-*tau*, were collected every 4-8 hours and raised at 16˚C. Considering the 2-2.5 times slower development at this temperature, this collection interval roughly corresponds to 2-4 hours at 25˚C. Staging of pupae was performed in the same way. To heat shock the animals, the culture bottles were soaked in a water bath for 30 minutes at 25-29˚C. After the heat shock the animals were allowed to develop at 16˚C. Adult flies were fixed on the day of eclosion in order to avoid the generation of clones by undesired Flippase activity frequently observed in later adult stages. This may be due to an enhancer-trap effect of the insertion in the hsp70-*flp* strain used in this study.

Under the conditions described above, 5-20 clones were labelled in each hemisphere of the central brain. In the MB region, less than 5% of the examined brains (in total approx. 1000) had labelled cluster(s). This means that the excision of the FRT cassette took place in only about one in 160 MBNbs. In the larval brain, only one preparation labelled two clusters (each with a labelled neuroblast) in the MB region; the other 12 had a single cluster. Thus, it is very likely that the MB cell clusters we observed in this study represent single clones.

Female brains were primarily used for documentation, although male brains were also examined for comparison. The vast majority of the clones were generated by giving a heat shock at 100 hours after larval hatching (corresponding to approx. 45-50 hours at 25˚C), when most of the postembryonic neuroblast in the central brain have become mitotically active (see Fig. 3 of Ito and Hotta, 1992). Although various types of clones were observed, only the clones in the MB region were analysed in this study. To study the temporal aspects of the MB development, a heat shock was given either just after larval hatching or 70-80 hours after puparium formation (at 16˚C).

Enhancer-trap strains

The Mz series GAL4 enhancer-trap strains were generated by Joachim Urban and coworkers of G. M. Technau's group at University of Mainz (Ito et al., 1995), and the Np series strains by us. Both series were made by crossing a pGawB strain (Brand and Perrimon, 1993) with the P[ry^{+}; Δ2–3] (99B) strain (Robertson et al., 1988). Screening for strains with MB-preferential GAL4 expression was performed by crossing the strains with UAS-*lacZ* (Brand and Perrimon, 1993) for X-gal staining. In the 19 strains used in this study, GAL4 expression was observed not only in the MB but also in various brain regions other than the MB. Since the expression patterns in these regions were different among strains, it is likely that these strains detect different enhancer activities. To reveal the structures of the GAL4-expressing cells in more detail, the strains were crossed with either UAS-*tau* (K. Ito, S. Schneuwly et al., unpublished) for immunohistochemistry or UAS-GFP S65T (B. Dickson, personal communication) for confocal microscopy. Female brains were used for presentation, though no sexual dimorphism was observed in these strains.

Partial ablation of MBNbs with hydroxyurea

The ablation of proliferating neuroblasts was performed as described by Prokop and Technau (1994). Newly hatched larvae were fed with medium containing HU for 3 hours, washed with water, and raised with standard medium thereafter. To increase the population of animals in which one or two MBNbs escape ablation, we used HU at a concentration of 3 mg/ml medium instead of 30-50 mg HU/ml as used by others (Prokop and Technau, 1994; de Belle and Heisenberg, 1994).

Staining of the brain

The dissection and staining of the brain have been described elsewhere (Ito et al., 1995). The mouse monoclonal anti-Tau primary antibody (Sigma, diluted at 1:500), biotin-conjugated secondary antibody (Vectastain, 1:500) and Vectastain Elite ABC kit for DAB staining were used to reveal cells labelled with Tau. Whole-mount preparations were embedded in araldite and kept in 0.3-0.35 mm capillaries, which can be rotated under microscope objectives (Prokop and Technau, 1993). To make pictures simulating a large depth of focus (Figs 3, 4), 10-50 photographs were taken at slightly different focal planes using a Progress 3012 digital scanning camera (Kontron) and an Axiophot

microscope (Carl Zeiss) with 63× Nomarski optics. Images were montaged digitally using Adobe Photoshop 3.0 software (Adobe) on a Macintosh computer (Apple).

For three-dimensional stereographs (Figs 5, 6), the GFP-labelled brains were dissected out, fixed with 4% formaldehyde for 50 minutes, washed several times with PBS, incubated with 50% glycerol for 1 hour, and mounted in 80% glycerol. Antibody staining of glial cells (Fig. 5) was performed by using the rabbit polyclonal anti-Repo antibody 4α3 (Halter et al., 1995; diluted at 1:100) and Texas Red-conjugated secondary antibody (Amersham, 1:100). GFP S65T retains its strong fluorescence even after the antibody staining. 160-200 serial optical sections at 0.4 μm or 1.0 μm intervals were taken using a Sarastro 2000 Z laser confocal microscope (objectives 40× and 100×, excitation 488 and 568 nm). Three-dimensional reconstruction was done by using the ImageSpace software (Molecular Dynamics).

The original images reported in this paper have been submitted to the FLYBRAIN image database under the accession number AB00150.

RESULTS

The MBNbs exclusively generate neurones and glial cells of the MB

A previous study with the BrdU-labelling method revealed that the progeny of the four MBNbs occupy the position expected for the Kenyon cells (Ito and Hotta, 1992). There are three possibilities regarding the behaviour of the individual MBNbs (Fig. 1). The first possibility is that each MBNb is specified uniquely to generate neurones that project to a specific region of the MB neuropile, e.g. the α lobe (model A). Another possibility is that each MBNb may not be limited to a specific substructure, but contribute to all of the cellular repertoire of the MB (model B). The last possibility is that the progeny of the MBNb are not confined to the MB, but may project to other regions of the brain (model C).

To distinguish between these possibilities, it was necessary to reveal all of the cytoplasmic structures of the clonally related cells. This was attained by a newly developed method that combines the yeast Flippase/FRT system (Golic and Lindquist, 1989; Struhl and Basler, 1993) with the GAL4/UAS system (Brand and Perrimon, 1993) (Fig. 2). The central component is called AyGAL4, which is a cytoplasmic Actin promoter-GAL4 fusion gene that is made non-functional by the insertion of transcriptional termination signals. In the initial state (Fig. 2A), transcription from the ubiquitous Actin promoter in AyGAL4 is terminated at the hsp70 transcriptional termination signal (Struhl and Basler, 1993). Upon heat shock, the hsp70 promoter drives the *flp* gene that encodes Flippase recombinase. The Flippase protein catalyses recombination at the two FRT sequences and removes the termination signal as well as a marker gene, *yellow* (Fig. 2B), generating a functional Actin promoter-GAL4 fusion gene (Fig. 2C). GAL4 protein expressed under the constitutive Actin promoter then activates the UAS-linked reporter gene *tau*. Thus, only the cells that experienced recombination, and their progeny, express the bovine Tau protein, which can be revealed using an anti-Tau monoclonal antibody. Since Tau is a microtubule-associated protein that is actively distributed throughout the cytoplasm via the slow transport mechanism, the whole cellular structure and the innervation pattern of the labelled cells can be resolved.

When the heat shock is given just after larval hatching, the clones of the MBNbs are preferentially labelled, as they are four of the only five neuroblasts mitotically active at this stage (Ito and Hotta, 1992). Fig. 3A-C shows one such clone stained at the end of the third instar larval stage. At the top of a tightly packed cluster of smaller Kenyon cells (KC), a single neuroblast (MBNb) is observed. Considering the fact that a neuroblast proliferates asymmetrically to generate ganglion mother cells that divide to form neurones (Bauer, 1904; Bate, 1976), it is very likely that the cluster of labelled cells in Fig. 3A-C represents a single clone (see also Materials and Methods).

Using Tau protein as a marker, the axonal projections from the MBNb progeny were visualised in their entirety. The calyx (ca) is seen below the cell cluster. At the root of the pedunculus, the fibres of all the cells form a single fascicle. The bundle de-fasciculates to form medial (M) and lateral (L) bundles after running one-third the length of the pedunculus. The medial bundle bifurcates at the frontal end of the pedunculus to form α and β lobes. The lateral fibres form the 'spur' (sp: also called 'knee' by Heisenberg et al., 1985) structure at the frontal end and bend inwards to the γ lobe. Some of the lateral fibres bend upwards to form the α′ lobe (also called 'wedge' by Heisenberg et al., 1985), which is located adjacent to the α lobe. Thus, the cells of a single clone apparently contribute to all the known

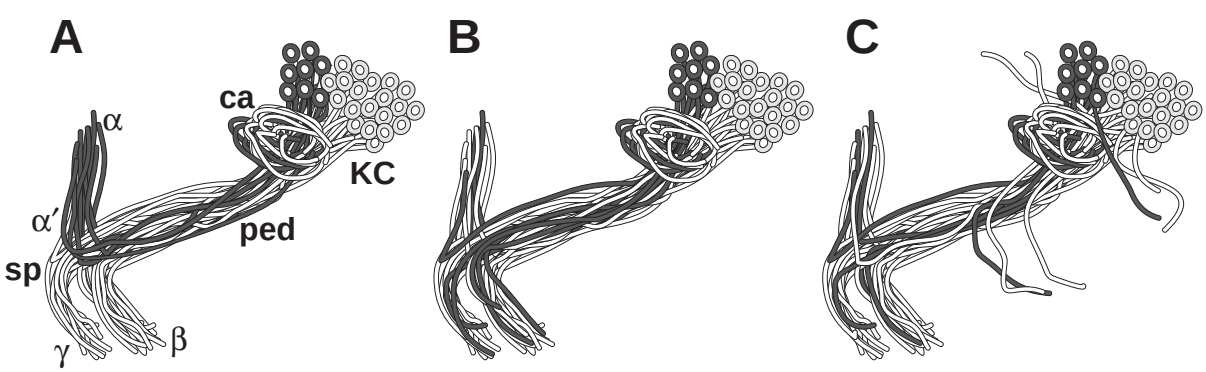

Fig. 1. Three models of the projection of the mushroom body neuroblast progeny. A mushroom body (MB) consists of a large cluster of Kenyon cells (KC). The axons form various neuropile structures called calyx (ca), pedunculus (ped), spur (sp) and α, α′, β and γ lobes. (A) The progeny of a particular mushroom body neuroblast (MBNb) project to a specific region of the MB neuropile, e.g. the α lobe (black cells). (B) A MBNb clone contributes to the entire cellular repertoire of the MB. (C) The progeny of the MBNb are not confined to the MB. Some neurones project to other regions of the brain.

MB substructures (model B in Fig. 1). It is also clear that the progeny of a MBNb innervate exclusively structures within the MB neuropile. These two features were consistent for all the MBNb clones examined, irrespective of their relative position in the cortex (*N*=13).

The insect brain experiences massive reorganisation during metamorphosis (Bodenstein, 1950; Restifo and White, 1991). The structure of the clones after metamorphosis, however, is surprisingly unchanged (Fig. 3D-F). The cell bodies are still tightly packed, and the fasciculation of the fibres persists even in the adult brain. As in larvae, a single clone contributes to the entire MB structure. Aside from their relative position in the cortex, no differences were observed among the clones (*N*=19). While the fibres in the medial bundle are tightly bound, those in the lateral bundle show a more diffuse structure (Fig. 3E). A small subset of the lateral fibres run dorsal, ventral, or even medial to the medial bundle before they project to the spur region.

Most of the cells in the clone have round-shaped cell bodies with single fibres running towards the neuropile, but some cells show irregular morphology with processes confined in the cortex (CBG in Fig. 3F). The position and morphology of these cells are very similar to the glial cells of the 'cell body glia' subclass (Ito et al., 1995). This suggests that the MBNbs give birth not only to neuronal Kenyon cells but also to the cell body glial cells.

Late generated neurones show a more restricted distribution in the MB

While all the other neuroblasts stop dividing between 20 and 30 hours after puparium formation (at 25˚C), the four MBNbs continue proliferating until very late in the pupal stage (Ito and Hotta, 1992). To analyse what structures are formed by the neurones generated during these later stages, we examined clones labelled at 70-80 hours after puparium formation (at 16˚C, equivalent to approx. 30-35 hours at 25˚C). A heat shock at this stage produces a tightly packed MB cell cluster near the brain surface, which forms a single medial bundle towards the α and β lobes (Fig. 3H, I). In addition, a few scattered MB neurones that project through the lateral bundle (L) to the γ lobe are labelled. Since these scattered cells are also labelled in brains that do not contain a MBNb clone (Fig. 3G), we infer that these cells were labelled either by postmitotic Flippase activity or by the division of the ganglion mother cells. It is very likely that most of the Kenyon cells in the late-induced clones innervate only the medial pedunculus bundle and the α and β lobes, but not the lateral bundle and the γ lobe.

GAL4 enhancer-trap strains reveal four almost identical cell sets in the MB

Since a heat-shock-induced single MBNb clone represents a random selection from the pool of four mitotically active MBNbs, the failure to observe any significant difference among a total of 32 single MBNb clones suggests that a group of cells produced by any of the MBNbs are nearly identical at the structural level. To study further the identity of these clones in terms of gene expression, we screened 488 GAL4 enhancer-trap strains for staining in the MB, and found 19 strains that label various subsets of the MB cells (Fig. 4). Surprisingly, all 19 strains revealed four clusters of cells in the MB cortex and four bundles of fascicles at the root of the pedunculus (Fig. 4, see also Fig. 5). The fact that not just a few but all the strains label the fourfold structure strongly suggests that each cluster contains all composite cell types labelled in these strains. In other words, the sets of cells in each of the four clusters are nearly identical.

In the strains that label only a small subset of Kenyon cells in the medial bundle (towards α and β lobes), the four clusters and fascicles are well separated (upper panels in Fig. 4). The number of clusters and fascicles are the same in the strains where a larger subset of Kenyon cells are labelled, although the border between the four is more obscure (lower panels in Fig. 4). The separation is less clear in the lateral bundle. This may reflect the structural difference between the two bundles (discussed above, see also Fig. 3E).

The four fascicles found in these strains may correspond to the four fibre bundles observed previously with the antibody fb45 (Bicker et al., 1993). In several enhancer-trap lines (i.e. Np 35, Mz 918, Mz 1081, Mz 1162 and Mz 529 of Fig. 4), two of the four fascicles in the pedunculus medial bundle fuse first as they project from posterior to anterior, forming two bundles that eventually fuse at a more anterior position. This situation is similar to that found in the enhancer-trap strains c739 and

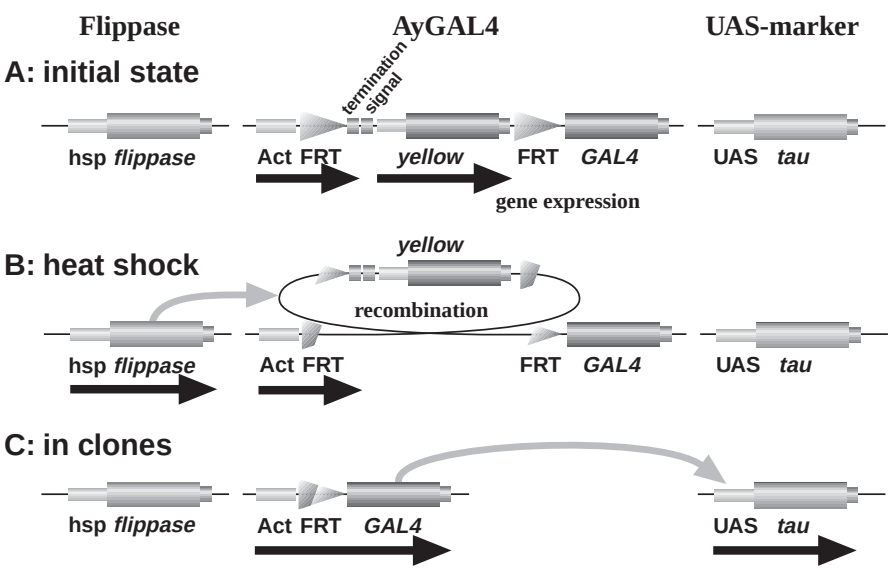

Fig. 2. The mechanism of clone labelling using the combination of flp/FRT and GAL4/UAS systems. (A) Before heat-shock treatment, the Act5C promoter-GAL4 fusion gene is interrupted by a FRT cassette containing transcriptional termination signals and thus remains silent. (B) Heat shock induces expression of the *flp* gene encoding the Flippase recombinase, which excises the FRT cassette from the AyGAL4 construct. (C) The GAL4 protein expressed from the rearranged Act5C promoter-GAL4 fusion gene activates transcription of the UAS-*tau* reporter gene. If the excision of the FRT cassette takes place in a proliferating neuroblast, all subsequent clonal progeny express the Tau protein.

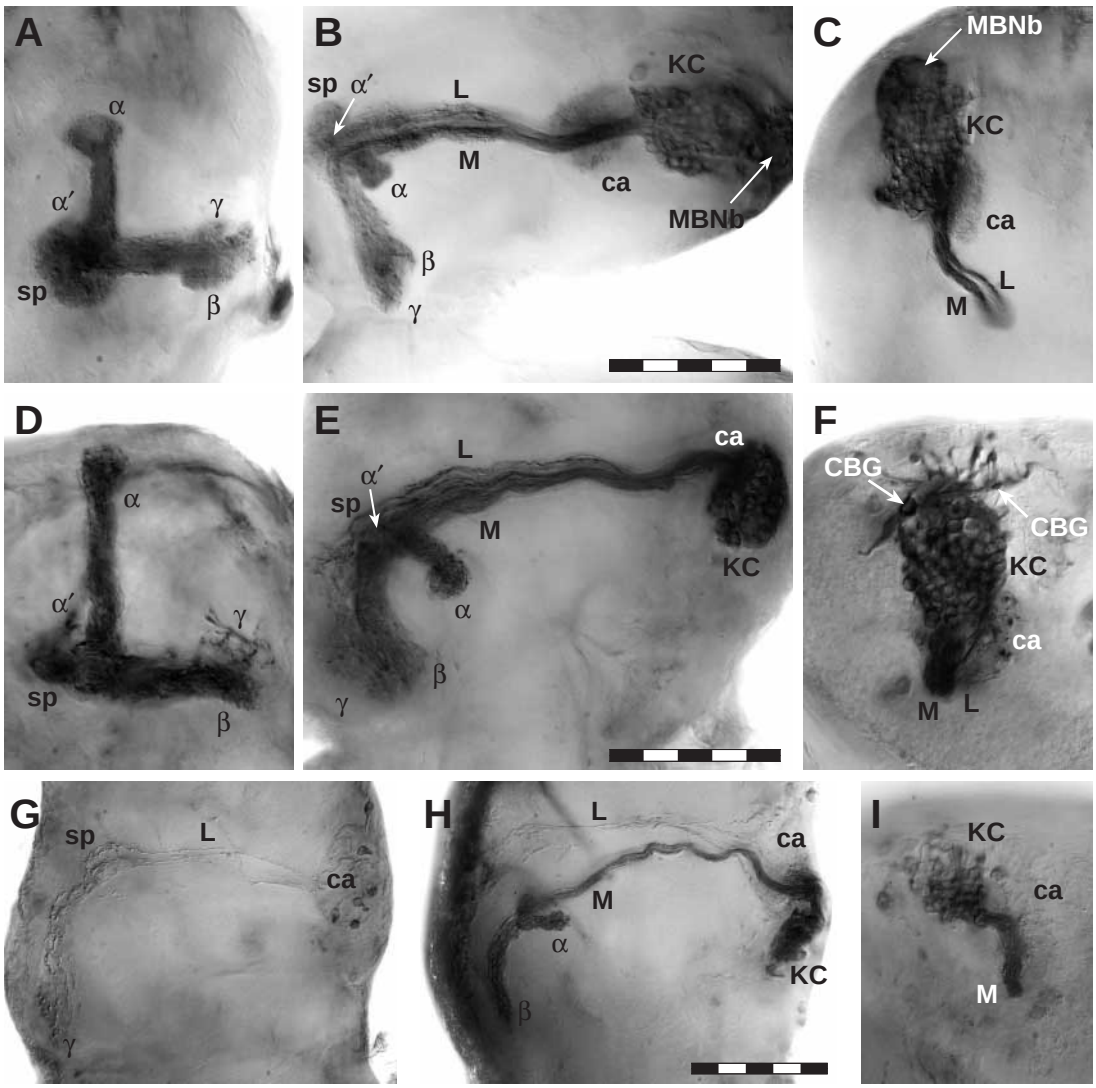

Fig. 3. MBNb clones labelled with the Flippase system. (A-C) A larval MBNb clone (induced just after hatching, stained at the end of the larval stage). Frontal (A), horizontal (B) and posterior (C) view. The α, α′, β and γ lobes, spur (sp), lateral (L) and medial (M) bundles of the pedunculus, calyx (ca), cell body clusters (KC) and the MBNb are seen. (D-F) An adult MBNb clone (induced at the end of the second instar, stained on the day of eclosion). Frontal (D), horizontal (E) and posterior (F) view. Note the irregular-shaped glial cells (CBG) in the cell body cluster of the Kenyon cells (KC) in F. (G-I) An adult MBNb clone labelled at the mid-pupal stage. A brain without any MBNb clones (G), with a clone (H) and the posterior view of the clone (I) are shown. Note that a much smaller number of cells are labelled than in clones induced at earlier stages (C and F). All figures are montaged from 20-50 photographs. Bars, 50 μm.

201y reported by Yang et al. (1995), suggesting that two of the four clusters are more closely associated with each other. This may explain why in some cases only two clusters have been described (e.g. strain c35 in Yang et al., 1995); the border between the two groups in each cluster might have been obscured when a very large subset of cells was labelled.

The spatial relationships between the clonal clusters and the fascicles were further studied using confocal microscopy (Fig. 5). When the GAL4 enhancer-trap expression patterns were visualised using UAS-GFP S65T (B. Dickson, personal communication, coloured green), four clusters of cell bodies (labelled with numbers 1 to 4) as well as four bundles of fascicles (numbers *1* to *4* in *italics*) running towards the pedunculus are observed. The four fascicles of fibres run at the ventralmost region of the calyx neuropile. Although there is no twist between the neighbouring fascicles, the alignment of the four fascicles and cell body clusters is not identical in different individuals (compare Fig. 5A and B). Glial cells (blue, labelled with the glial-specific anti-Repo antibody: Halter et al., 1995) are observed both in the cell body clusters and between the four fascicles. Of the two classes of these glial cells, only the former are labelled in the Flippase-mediated clones (see Fig. 3F).

A single MBNb is sufficient to generate autonomously all of the MB structures

Our results suggest that the cells in a single MBNb lineage contribute to all of the MB substructures in the intact brain. It is possible that each of the four MBNbs has identical potential to produce the complete MB structure. Alternatively, one or a few MBNb progeny may perform a pioneering function in guiding axons from other MBNb lineages. To address this question, we performed partial ablation of the MBNbs and analysed the projection patterns of cells labelled with GAL4 enhancer trap in animals that have only one of the four MBNbs. To selectively ablate the MBNbs, newly hatched larvae were fed medium containing 3 mg/ml of HU for 3 hours. Under these conditions, 20-30% of examined brains had only one (or two) surviving MBNb(s) in either one or both hemisphere(s) (*N*=30-60, depending on the marker strains). (Even in the brains without any surviving MBNbs, a thin fibre bundle was observed in some strains. This is probably due to the embryonic Kenyon cells generated before the ablation. See also de Belle and Heisenberg, 1994.)

The strain Mz 717 labels fibres in the dense α and β lobes, as well as fibrous γ and α′ lobes. Four clusters of cell bodies

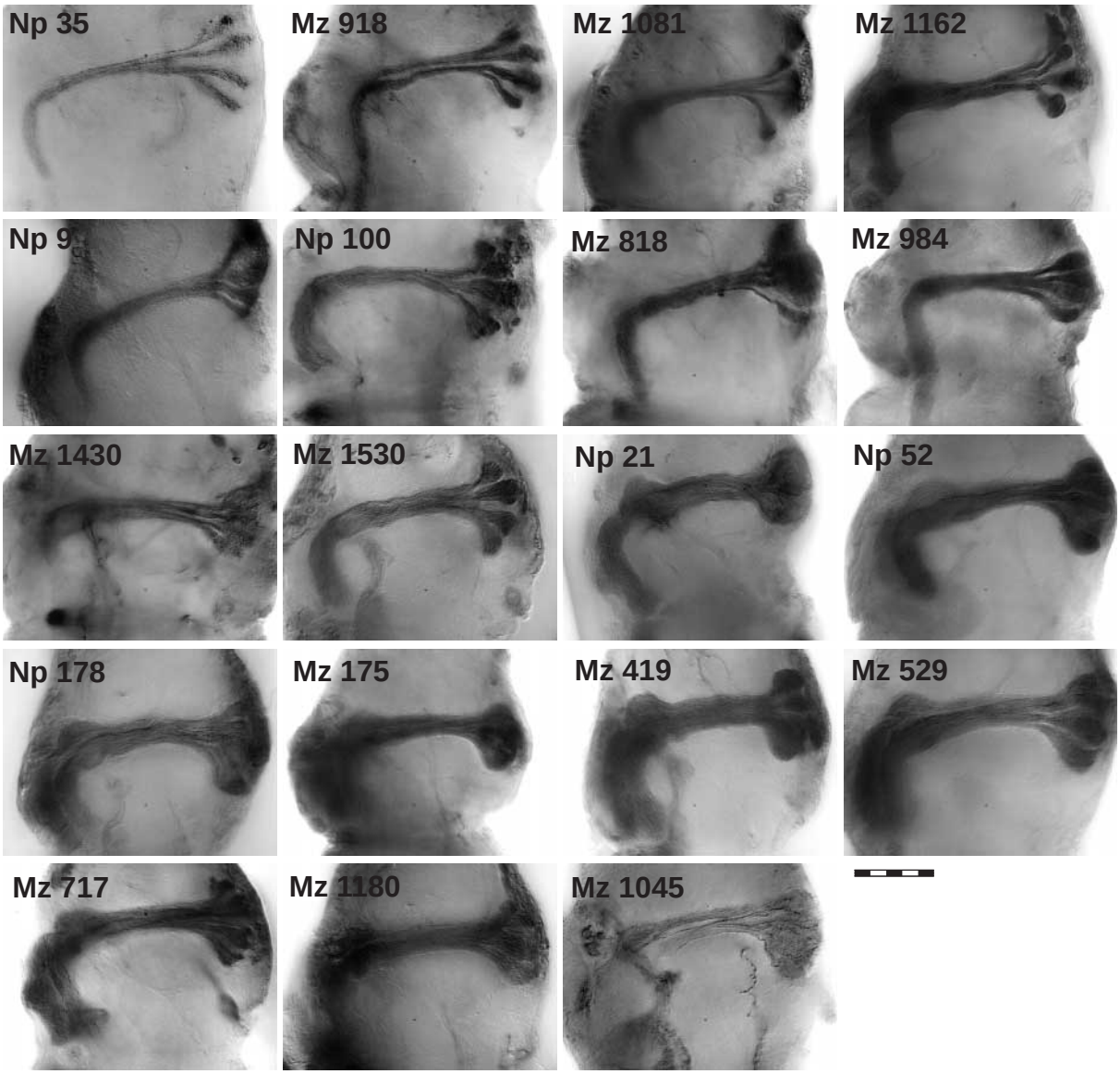

Fig. 4. GAL4 strains that label subsets of mushroom body neurones. The GAL4 expression was detected using UAS-*tau*. Rows 1 and 2 (from the top) and the left half of row 3 show the strains that label neurones that project to the medial bundle. The right half of row 3, row 4 and the left half of row 5 show the strains that label a larger number of neurones in both medial and lateral bundles. Most strains show clear separation between the clusters. The border between the clusters is less clear in Mz 1045 of row 5, which labels only a few scattered neurones that innervate the lateral bundle and the γ lobe. All figures are montaged from 10-30 photographs. Bar, 50 μm.

are observed in the intact brain (Fig. 6A). In the brain that has only one surviving MBNb clone in each hemisphere (Fig. 6B), only one cluster is seen in the cortex. Although the fibre number is greatly reduced, the clone forms essentially identical structures in the lobe region as would four clones in untreated animals. However, we found that the β lobes of both sides often fuse together in the HU-treated flies (arrowhead in Fig. 6B).

The strain Mz 984 labels fibres in the core region of only α and β lobes. The large round tip of the α lobe and the split tips of the β lobe (two arrowheads in Fig. 6C) are labelled in the intact brain. The same characteristic structure can be observed in brains with single surviving clones (Fig. 6D). Like Mz 717, the β lobes of the HU-treated animals are fused with each other. In a brain that has only one MB clone, the β lobe fibres seem to divert after crossing the midline (Fig. 6E). This would suggest that these cells extend axons in unusual directions to compensate for the reduced number of fellow neurones. It is important to note that only a defined substructure was affected by the HU treatment. Although the fibres in the upper tip of the β lobe seem to cross the midline (arrowhead in Fig. 6D), the γ lobe and the lower tip of the β lobe look intact. Thus, we think that the fused lobe is a secondary outcome rather than a direct consequence of the HU-mediated ablation. Except for this fusion, the lobe and pedunculus structure of the single clones is essentially identical with that of the intact brain.

With all the MB subtype markers examined, the labelled structure of the surviving clones was essentially identical to the staining pattern of the intact brain, except for the reduced cell

numbers and the fused lobes (Fig. 6 and data not shown). Considering that the escape from the ablation should occur randomly, these results strongly suggest that a single MBNb, in the absence of the other MBNbs, can generate all the substructures labelled with these markers.

DISCUSSION

Cell-lineage analysis in the adult brain

In this study, we analysed clones of the *Drosophila* MBNbs. This is the first attempt to perform cell-lineage analysis in the adult brain. To achieve this, we needed an efficient method to introduce a stable lineage tracer. Though enhancer-trap strains and promoter-*lacZ* constructs may in some cases label most descendant cells of a given precursor cell (e.g. Jacobs et al., 1989), they are not likely to label an entire cell lineage throughout development, since such markers rely on the expression pattern of certain genes. Markers such as DiI label all the clonally related cells made during embryogenesis (Bossing and Technau, 1994). The single cell neuroblast transplantation technique (Technau, 1986) has also been used in cell-lineage analysis of embryonic nervous system. Unlike such genetic labelling, tracer chemicals introduced into precursor cells will be diluted below the detection level during postembryonic development as a result of cell division and growth (Prokop and Technau, 1991). Although clones can be labelled by transfecting with genetically manipulated retrovirus in vertebrates (Turner and Cepko, 1987; Luskin et al., 1988), such viruses are not readily available in *Drosophila*.

The Flippase-mediated clone labelling system was first introduced to study cell fates in imaginal discs (Struhl and Basler, 1993) and then used for cell lineage analysis in the embryonic peripheral and central nervous systems (Brewster and Bodmer, 1995; Buenzow and Holmgren, 1995). Since this technique requires only a simple heat shock to generate mosaics, it is possible to label clones in many individuals en masse at any developmental stage; this is a vital requirement for clonal analysis in the adult. While previous studies relied on *lacZ* to be activated upon recombination, we used the *gal4* gene in the FRT construct. This gives us the ability to use not only UAS-*tau*, as in this study, but also other UAS-linked reporter genes that label different intracellular structures. This system can also be combined with various UAS-driven effector genes to perform misexpression or ablation studies of clonally related cells that are simultaneously labelled with an appropriate marker gene.

Using the FRT-GAL4 system, we showed the following features of the *Drosophila* MB organisation. (1) The projection of the MBNb progeny is confined to the MB neuropile; (2) specific MB substructures are generated from individual MBNbs in a birth date-specific manner; (3) the postembryonic progeny of any of the four MBNbs contributes to all the known substructures of the MB, rather than each MBNb being specialised to produce certain MB substructures. In the following we discuss these points in turn.

Clonal origin of the MB

Clones induced just after larval hatching reveal that the progeny of a single MBNb project their axons to the entire MB neuropile. Our lineage analysis, however, does not address whether the MB structure is derived exclusively from the progeny of the four MBNbs. Each Flippase-mediated clonal cluster occupies as much as 20-30% of the MB cortex. The cell bodies are so tightly packed that there is little space for cells of other lineages lying within the cluster (see Fig. 3C, F). Ablation of all four of the MBNbs causes the total loss of the MB mass except for small remnants that could be attributed to the embryonic MB cells (de Belle and Heisenberg, 1994). A number of GAL4 enhancer-trap strains, however, do show a few neurones that send axons along the pedunculus (Ito et al., unpublished observation). Since their cell bodies do not lie in

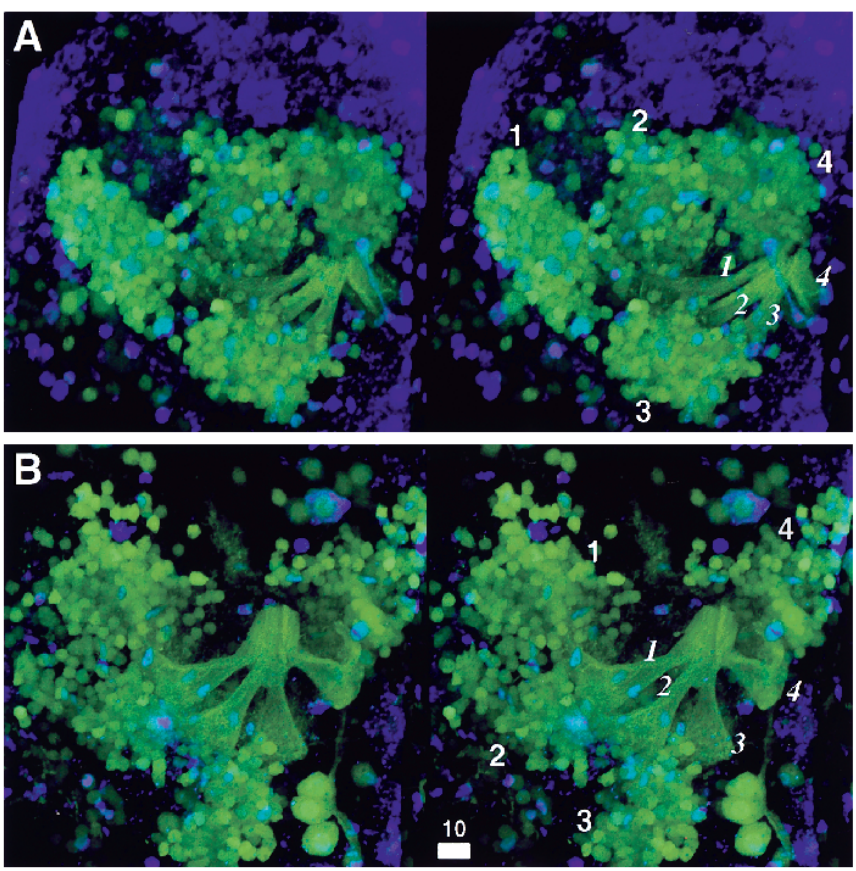

Fig. 5. Spatial relationship between the four fascicles, clusters and glial cells. Stereoscopic images of GAL4 enhancer-trap expression patterns (oblique view from the dorsoposterior). GAL4 strains Np 178 (A) and Mz 1530 (B) crossed to UAS-GFP S65T (green). Glial cells were labelled by the anti-Repo antibody (blue). In these strains no GFP-positive cells in the MB clusters are double-labelled with the antibody. Note that the GAL4 strains also label certain cells outside the MB, some of which are double-labelled. Three-dimensional reconstructions were made from about 200 confocal 0.4 μm optical sections. Bar, 10 μm.

the MB cortex above the calyx, we do not think that such cells can be called Kenyon cells. These results suggest that at least the vast majority of the MB structures are made by the cells of the four MBNb clonal units.

A MBNb gives birth not only to various types of Kenyon cells, but also to glial cells. This is the first finding that postembryonic neuroblasts are still able to generate two types of cells. A similar diversity of cell fates from a single cell lineage has been observed frequently in the embryonic ventral nerve cord. The NB 1-1, for example, generates glial cells, motor neurones and interneurones during only 10 hours of embryonic proliferation (Udolph et al., 1993; Bossing et al., 1996), while MBNbs proliferate for more than 200 hours (at 25°C) (Ito and Hotta, 1992).

There is a large discrepancy between the number of Kenyon cells counted in the adult brain (2500 per hemisphere on average, Hinke, 1961; Technau, 1984; Balling et al., 1987; Heisenberg et al., 1995) and the total number of MBNb progeny calculated from the estimated neuroblast cell cycle period (800-1200 per hemisphere, Ito and Hotta, 1992). Though we were not able to count the exact number of cells in the Flippase-mediated clones, there were substantially more cells than the estimated number of postembryonic MBNb progeny (216 per clone by Ito and Hotta, 1992). Thus, it is likely that the previous calculation was an underestimate. Some of the assumptions required for the calculation, e.g. the number of divisions of the ganglion mother cells, might not have reflected the real situation.

A correlation between the MB substructure and the birth date of the constituent neurones

Although clones generated at larval hatching project to the entire MB structure, the late-generated clones contain MB neurones that preferentially contribute to the α and β lobes (Fig. 3H). Such correlation between birth date and cell fate has been found both in insects and vertebrates. Although cell lineage appears to play a role in cell fate determination in the grasshopper CNS (Goodman and Spitzer, 1979; Doe and Goodman, 1985), in the vertebrate cortex such time dependency has been shown to be due to developmental changes in the microenvironment (McConnell, 1989). Further studies would be necessary to determine whether the projection pattern of the Kenyon cells is governed by their birth order or by extrinsic factors.

Even after the massive reorganisation of the brain structure during metamorphosis, the clonally related cells in the MB

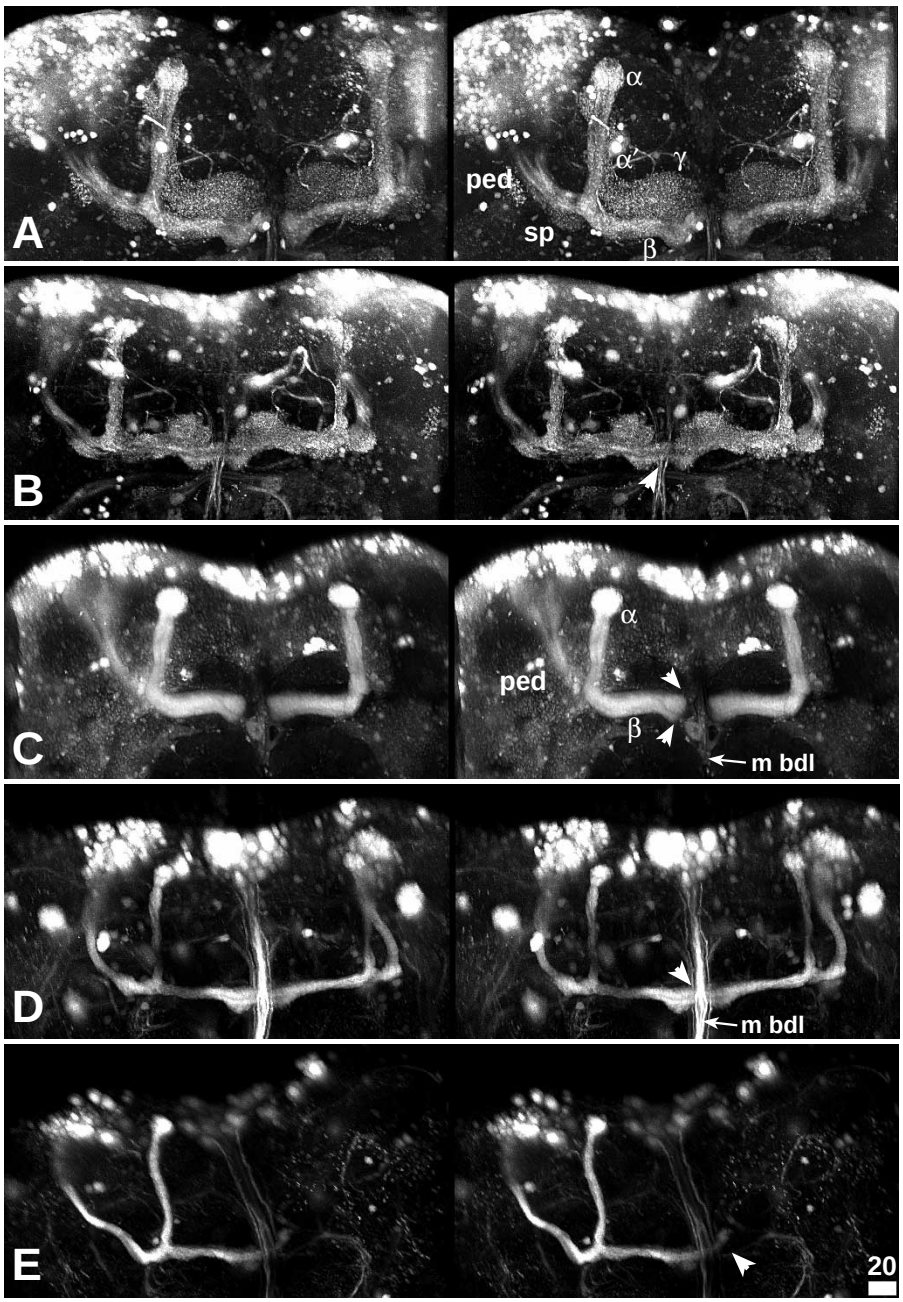

Fig. 6. Partial ablation of MBNbs with hydroxyurea. Stereoscopic images of GAL4 enhancer-trap expression patterns in brains where MBNbs were partially ablated with hydroxyurea (HU) treatment (oblique view from the anterior). (A,B) GAL4 strain Mz 717 crossed to UAS-GFP S65T. An intact brain (A) and a HU-treated brain with a single remaining MBNb clone in both hemispheres (B). Arrowhead in B indicates the fused β lobes. (C-E) GAL4 strain Mz 984 crossed to UAS-GFP S65T. An intact (C) and a HU-treated brain with a single remaining MBNb clone in both hemispheres (D) and one in only the right hemisphere (E). Arrowheads in C indicate the characteristic split tips of the β lobe and arrowheads in D and E indicate the β lobe fibres of the upper tip that cross the midline. Three-dimensional reconstructions were made from about 160 confocal 1 μm optical sections. α, α′, β, γ: lobes; ped, pedunculus; sp, spur; m bdl, median bundle. Bar, 20 μm.

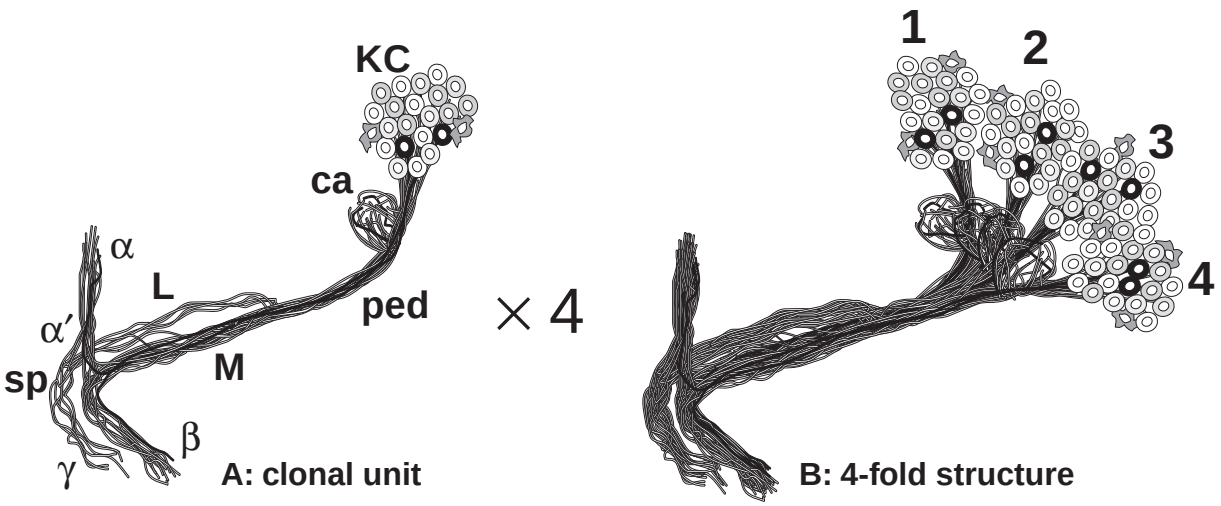

Fig. 7. Summary of the structural organisation of the mushroom body. (A) Each MBNb generates a clonal unit that contains glia and all the types of Kenyon cells (KC, in different shades of grey). After arborizing in the calyx (ca), the fibres make the pedunculus (ped) that defasciculates into the lateral (L) and medial (M) bundles. Fibres in the medial bundle form the α and β lobes, while those in the lateral bundle contribute to the spur (sp) and α′ and γ lobes. Flippase-mediated clones reveal such clonal units (Fig. 3). Partial ablation experiments show that any of the MBNb can autonomously generate such a unit even when all other MBNbs are absent (Fig. 6). (B) The intact MB is a fourfold structure of these clonal units. Since each unit contains all the cell types, a marker that detects a certain subset of cells (black) will reveal cells in all the four clusters (cf. Figs 4, 5).

extend axons via a single fascicle. The late-generated neurones also follow this route. When the MB fibres are drastically reorganised during early pupal stages, the Kenyon cells send out new fibres, apparently along the scaffold of persisting fibres (Technau and Heisenberg, 1982). Thus, it is likely that fasciculation is critical for axon guidance, not only in embryos (cf. Goodman and Doe, 1993) but also in the adult nervous systems.

A single MBNb lineage is a structural unit of the entire MB

Given the existence of four precursors that give rise to the MB (Ito and Hotta, 1992; this work) and the discrete structural subunits of the MB neuropile, it was possible that each MBNb is specialised to produce neurones that project to a specific substructure (model A in Fig. 1). Our clonal analysis indicates that the *Drosophila* MB is not produced by such substructure-specific or cell-type-specific precursors, but rather that each MBNb contributes to most, if not all, cell types found within the entire MB (model B in Fig. 1). We also showed that the four MBNb clones are essentially identical in structure and in gene expression patterns. Our observation that a single MBNb can generate autonomously a whole set of MB substructures is also consistent with the notion that the MB consists of fourfold repeated clonal units (summarised in Fig. 7). While the total number of brain neuroblasts shows a certain variability (85±7, Ebens et al., 1993), the number of MBNbs is remarkably constant (Ito and Hotta, 1992). This suggests that the recruitment of MBNbs in the embryonic brain is strictly regulated.

We cannot exclude the possibility that subtle differences between the four MBNb lineages were overlooked in our study. Although we found several hundreds of cells per clone, we were not able to determine the exact cell number, due to the tight packing of the cell bodies in the cortex. Thus, quantitative comparison among clones was not possible. Likewise, in certain GAL4 marker strains the four labelled clusters might actually have small quantitative differences that escaped our detection. There is also the possibility that a yet unknown marker, be it an enhancer-trap strain or antibody, can detect certain differences in the future.

Despite such reservations, it is obvious that the four clones are qualitatively virtually identical. The MB has a much denser cell population than any other brain region (Strausfeld, 1976). The Kenyon cells form an elaborate network array in the pedunculus and lobes. The abundance of these cells may be important in attaining a high level of resolution of input signals that convey various olfactory and other sensory information. Constructing such complex circuits as a quadruple structure of clonal units, each of which contains a complete set of MB cell types, might have been an efficient and economical way during evolution to improve the performance of information processing without fundamentally altering the process of MB development.

However, this does not necessarily mean that the four clonal units are also identical in their function. Since the four clones occupy different parts in the calyx, it is possible that the incoming neurones, such as the antennal lobe interneurones via the inner antenno-cerebral tract (also called antenno-glomerular tract), innervate the cells of these clones differently. Such input differences would reflect differences in their roles in information processing. Computer simulation suggests that even if there is no initial difference in input signals and neuronal connectivity, structurally identical neurones in a network array can develop different functions after a session of ‘training’ (Rumelhart et al., 1986; Lehky and Sejnowski, 1988). This sort of plasticity may well play an important role in the MB, since the volume of the neuropile, and thus the degree of complexity in the synaptic network, vary greatly according to post-eclosion experiences of each individual (Durst et al., 1994; Heisenberg et al., 1995).

Using Flippase-mediated clonal analysis and an array of GAL4 enhancer-trap strains, we demonstrated, for the first time, that the adult *Drosophila* MB represents a quadruplicate structure originating from four MBNbs, each of which autonomously produces a practically identical set of neurones and glial cells. This result is especially useful for understanding higher-order brain function, given the importance of the MB, and *Drosophila* itself, as model systems for complex behaviour. Moreover, since the *Drosophila* brain has at most 85 neuroblasts (i.e. 85 clonally related structures), mapping the clonal organisation of the entire central brain using the Flippase system is feasible. Such projects should provide us with fundamental insights into how the mature brain is constructed.

We thank Joachim Urban and Gerhard Technau for Mz series enhancer-trap strains and the anti-Repo antibody, Barry Dickson for the UAS-GFP S65T strain, Gary Struhl for plasmids with the FRT cassette, and Stephan Schneuwly and Heinz Sass for jointly generating the UAS-*tau* strain. We also thank Maiko Ue and Tsuyoshi Otsuka for the screening of GAL4 enhancer-trap strains, and Steve West for injecting the AyGAL4 construct. We are grateful to J. Steven de Belle, Martin Heisenberg, Cahir O'Kane, Andreas Prokop and Hasel Smith for fruitful discussion, and Gerhard Technau, Eric Nilsson and Steve West for critically reading the manuscript. A major part of this work was done at the ERATO Yamamoto Behaviour Genes Project. Contribution by Y. H. was supported by grants from the National Institute of Health (RO1 NS29662) and the Human Frontier Science Program. Y. H. was a Pew Scholar in Biomedical Sciences.

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(Accepted 9 December 1996)