Development of the *Drosophila* mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast

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SUMMARY

The mushroom bodies (MBs) are prominent structures in the *Drosophila* brain that are essential for olfactory learning and memory. Characterization of the development and projection patterns of individual MB neurons will be important for elucidating their functions. Using mosaic analysis with a repressible cell marker (Lee, T. and Luo, L. (1999) *Neuron* 22, 451-461), we have positively marked the axons and dendrites of multicellular and single-cell mushroom body clones at specific developmental stages. Systematic clonal analysis demonstrates that a single mushroom body neuroblast sequentially generates at least three types of morphologically distinct neurons. Neurons projecting into the γ lobe of the adult MB are born first, prior to the mid-3rd instar larval stage. Neurons projecting into the α' and β' lobes are born between the mid-3rd instar larval stage and puparium formation. Finally, neurons projecting into the α and β lobes are born after puparium formation. Visualization of individual MB neurons has also revealed how different neurons acquire their characteristic axon projections. During the larval stage, axons of all MB neurons bifurcate into both the dorsal and medial lobes. Shortly after puparium formation, larval MB neurons are selectively pruned according to birthdays. Degeneration of axon branches makes early-born (γ) neurons retain only their main processes in the peduncle, which then project into the adult γ lobe without bifurcation. In contrast, the basic axon projections of the later-born (α'/β') larval neurons are preserved during metamorphosis. This study illustrates the cellular organization of mushroom bodies and the development of different MB neurons at the single cell level. It allows for future studies on the molecular mechanisms of mushroom body development.

Key words: Mosaic analysis, Neurogenesis, Metamorphosis, *Drosophila*, Mushroom body, Neuroblast

INTRODUCTION

Information processing and storage are two fundamental brain functions. Proper function of the brain relies on the establishment of complicated networks among different types of neurons. Mapping this cellular organization is important in understanding the molecular basis of specific brain functions, such as learning and memory. A variety of techniques have been developed to resolve the neuronal networking at the single cell level. In particular, examining the entire morphology of random neurons using Golgi impregnation material demonstrates the highly diverse and intricate nature of neuronal morphology (Cajal, 1911). Other methods for visualizing individual neurons in an intact brain involve anterograde, retrograde or intracellular labeling (for review, see Cowan, 1998). These tools are invaluable in discovering complicated neuronal morphologies and their connections. However, such anatomical studies provide little insight into the molecular mechanisms underlying neuronal morphogenesis. Another important progress in understanding cellular organization of the nervous system is lineage analyses using recombinant retroviruses (for reviews, see Sanes, 1989; Cepko

et al., 1998). Although this approach has revealed important principles in the generation of diverse and characteristic neurons, effective means of studying the underlying mechanisms in a physiological environment are lacking. Investigating development of the *Drosophila* brain using powerful molecular genetic tools may shed new light on the mechanisms that govern the development of more complex nervous systems.

Several lines of evidence have implicated the mushroom bodies (MBs) of the *Drosophila* central nervous system (CNS) in olfactory learning and memory. For instance, chemical ablation of the MBs impairs olfactory learning and memory (de Belle and Heisenberg, 1994). Mutants with altered MB structure are defective in olfactory learning (Heisenberg et al., 1985). In addition, genes known to be necessary for olfactory learning and memory are preferentially expressed in the MBs (Crittenden et al., 1998, and references therein). Furthermore, the gross morphology of the MB is plastic and this morphological plasticity reflects the degree of stimulation in the living environment of individual animals (Technau, 1984; Heisenberg et al., 1995). To elucidate the molecular mechanisms for MB-mediated brain functions, it is important

to understand the cellular organization of the MB and its development.

The MBs appear as paired neuropils in the *Drosophila* brain (Fig. 1A). The gross morphology can be divided into discrete anatomical domains. The cell bodies are clustered at the dorsal posterior surface of the central brain and their dendrites form the calyx structure right below the cell body region. The axons form the peduncle, which extends ventrally toward the anterior surface of the brain, where it segregates into five terminal lobes (Crittenden et al., 1998) (Fig. 1B). The α and α' lobes project toward the dorsal surface, while the β, β' and γ lobes project toward the midline of the brain. As revealed by Golgi staining, MB neurons are unipolar, their dendrites branch into the calyx right below the cell body, and individual axons project through the peduncle and extend into the lobes. In the housefly, two types of axonal projections can be distinguished based on their branching patterns (Strausfeld, 1976). Some axons bifurcate into dorsal and medial branches, while other axons only extend toward the midline without bifurcation. MB neurons with these stereotyped projection patterns have also been described in the *Drosophila* (Yang et al., 1995; Armstrong et al., 1998).

Interestingly, these five lobes can be grouped into three sets based on the expression levels of various MB-enriched antigens (Crittenden et al., 1998). For any given antigen, α and β lobes always have comparable expression levels, α' and β' lobes always share strong similarities, and the γ lobe is distinct from the others. These findings have led to the proposal that there are three major projection configurations of MB axons. One type of MB neuron projects its axons only into the γ lobe, the second type projects its axon branches into both α' and β' lobes, while the third type projects its axon branches into both α and β lobes (Crittenden et al., 1998).

In each brain hemisphere, the MB is derived from four neuroblasts (Nbs). Labeling DNA replication by BrdU incorporation revealed continuous proliferation of MB Nbs from the embryonic stage to the late pupal stage (Truman and Bate, 1988; Ito and Hotta, 1992). Interestingly, each MB Nb generates a similar set of neurons and glial cells, as evidenced by the 4-fold organization of marked neurons in various MB enhancer trap lines and the same gross morphology of marked neurons derived from random MB Nbs (Ito et al., 1997). Therefore, how MB neurons with different axonal projections

Fig. 1. The organization of the adult mushroom bodies (MBs) and clonal analysis using MARCM. (A) Composite confocal images of an adult brain show the morphology of the paired MBs, one in each brain hemisphere. Expression of the *mCD8-GFP*, driven by the *GAL4-OK107*, allows for visualization of the whole MBs. seg, supraesophageal ganglion; ol, optic lobe; sub, subesophageal ganglion. (B) Close-up view of the right MB in A. Five axonal lobes are grouped into three sets, based on a previous proposal (Crittenden et al., 1998). The γ lobe is outlined in red, the α' and β' lobes are outlined in green, and the α and β lobes are outlined in blue. (C) Schematic drawing shows the essence of the MARCM system. Mitotic recombination between two FRT sites (triangles) results in loss of the repressor transgene (*tubP-GAL80*) in one of the daughter cells, and hence GAL4-dependent expression of the marker transgene (*UAS-mCD8-GFP*). (D) Schematic drawing shows how mitotic recombination in a dividing Nb can lead to formation of two mutually exclusive types of marked clones. If the regenerated Nb loses the repressor gene, all postmitotic neurons generated subsequently in the same lineage will be labeled (upper). In contrast, if the GMC loses the repressor gene, only two neurons derived from this GMC will be labeled in the whole lineage (lower). In addition, mitotic recombination in a dividing GMC can generate a single cell clone independently (lower). (E) Composite confocal images of an adult MB Nb clone demonstrate five axonal bundles, three projecting medially and two projecting dorsally. This clone was generated by inducing mitotic recombination in a newly hatched larva with the genotype of *hs-FLP/Y; FRT^{G13},tubP-GAL80/FRT^{G13},UAS-mCD8-GFP; GAL4-OK107/+*. (F) Based on the observation that five lobes could be grouped into three sets of bundles with characteristic antigen compositions, Davis and his colleagues proposed that there are three types of neurons with different characteristic axonal projections (Crittenden et al., 1998). One type of neurons (red) project their axons only into the γ lobe, another type of neurons (green) have branched axons projecting into both the α' and β' lobes, and the third type of neurons (blue) have branched axons projecting into both the α and β lobes. The question is: how are they generated from a single neuroblast? In this and all subsequent figures, the brain is shown in an oblique configuration so that dorsal-anterior is up. All figures except Fig. 1A have midlines on the right side. The unit in the scale bar is μm in this and all subsequent figures.
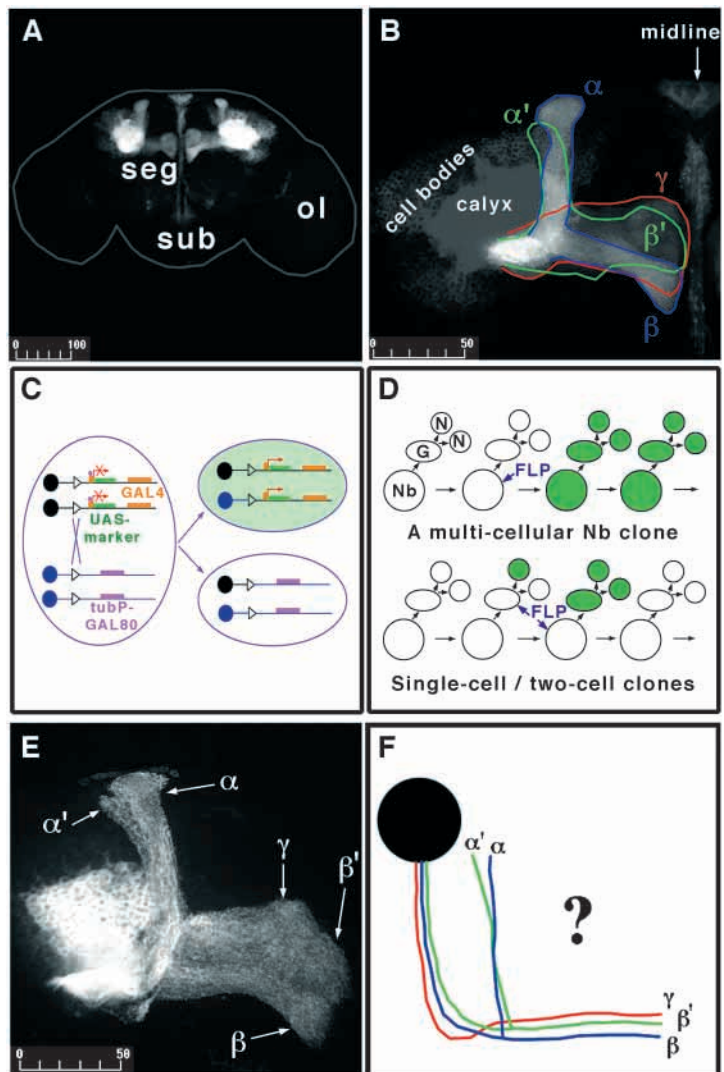
are derived from the same Nb remains to be elucidated (Fig. 1F). Another interesting aspect regarding development of the MB is reorganization of larval MB neurons during metamorphosis (Technau and Heisenberg, 1982). In the CNS, there are three known fates for most larval neurons during metamorphosis. Larva-specific neurons are removed by programmed cell death after puparium formation. Adult-specific neurons continue their morphogenesis through the pupal stage. In addition, some larval neurons that persist into adulthood withdraw their larval processes and extend new adult-specific processes during metamorphosis (Truman, 1990). Most MB neurons persist through metamorphosis and thus it is important to determine how MB neurons born at different stages reorganize their processes during metamorphosis.

We have recently developed a system for mosaic analysis with a repressible cell marker (MARCM; Lee and Luo, 1999). As summarized in Fig. 1C, the system starts with cells heterozygous for a transgene encoding a repressor (the yeast GAL80 protein that inhibits the activity of the transcription factor GAL4). The repressor gene is removed from one of the daughter cells after a FLP/FRT-mediated mitotic recombination, thus allowing marker expression (under the control of GAL4) specifically in this daughter cell (green) and all its progeny. When applied to the CNS of *Drosophila*, the division pattern of which is illustrated in Fig. 1D, one can generate Nb, 2-cell, or single-cell clones depending on when mitotic recombination occurs and which cell loses the repressor. By controlling the timing of mitotic recombination with a heat-shock promoter-driven FLP recombinase transgene, all three types of clones can be generated at a desired developmental stage and examined later.

By applying the MARCM system to study MB development, we show here that a single MB Nb can generate three distinct classes of neurons in a sequential fashion. Corresponding to their axonal projection patterns, they are referred to as γ neurons, α′/β′ neurons and α/β neurons. The transitions made to produce different neurons, both from γ to α′/β′ neurons and from α′/β′ to α/β neurons, occur abruptly and completely. Further, we show contrasting behaviors of γ and α′/β′ neurons during metamorphosis. These results shed light on the functions of these different classes of neurons and lay the ground plan for systematic analyses of gene function in different aspects of MB development.

MATERIALS AND METHODS

Construction of fly strains used in this study

A *hs-FLP* transgene on the X chromosome was assembled with a second chromosome containing both *FRT^{G13}* (2R) and *tubP-GAL80* transgenes to generate the *hs-FLP; FRT^{G13},tubP-GAL80* stock using standard genetic techniques. The fourth chromosome containing the *GAL4-OK107* enhancer trap (Connolly et al., 1996) was assembled with a second chromosome containing both *FRT^{G13}* and *UAS-mCD8-GFP* transgenes to generate the *FRT^{G13},UAS-mCD8-GFP; GAL4-OK107* stock. The recombinant chromosome containing *GAL4-201Y* (Yang et al., 1995), *FRT^{G13}* and *UAS-mCD8-GFP* was constructed and confirmed by GFP expression. The remaining stocks used in this study have been described previously (Lee and Luo, 1999).

Fly culture, histology and microscopy

Drosophila melanogaster were grown on standard media at 25°C. To induce mitotic recombination, staged larvae, which had hatched within a 2-hour interval, were collected into vials containing about 10 ml of regular fly food at the density of 80 larvae per vial, heat shocked in a 37°C water bath for 40 minutes and then returned to 25°C. To avoid complications resulting from sex-specific differences in the neurogenesis of the MB, we restricted our analyses to male organisms for most of the studies. At certain developmental stages, central nervous tissues were processed for GFP fluorescence or immunofluorescence microscopy according to procedures described previously (Lee and Luo, 1999). For anti-fasII staining, mAb1D4 was used at 1:10. Images were taken with a Bio-Rad MRC 1024 laser scanning confocal microscope using the Laser Sharp image collection program.

Quantitative analysis of the cell bodies present in MB Nb clones

After heat shock at various early developmental stages, male adult flies, younger than 5 days old, were dissected and their brains were examined for marked MB Nb clones. A total of 11 MB Nb clones were selected for quantification of the volumes occupied by MB cell bodies. Four random clones, representing those Nb clones having all five full MB lobes, were selected from the brains heat shocked right after larval hatching (ALH). Among the brains heat shocked around 3.5 days ALH, four clones containing the largest β′ lobes were selected to represent Nb clones with four full MB lobes. As representative Nb clones with full α and β lobes, three clones containing only one or two processes in the β′ lobe were selected from the brains heat shocked around puparium formation. For each clone, Z-series confocal images were collected at a 1 μm step to cover the whole cell body region. On each focal plane, the cell-body-containing region was defined manually and its area was measured using the Laser Sharp processing program. The volume of the whole cell body region was calculated by multiplying every area by 1 μm to get the volume for each focal plane and then adding all volumes together.

RESULTS

Marking the entire morphology of mushroom body (MB) clones using MARCM

The adult MBs, as visualized by a MB GAL4 line *GAL4-OK107* (Connolly et al., 1996)-dependent expression of a membrane-targeted mCD8-GFP (Lee and Luo, 1999), appear as paired neuropils with characteristic structural features, including four clusters of cell bodies, the calyx, the peduncle and the lobes (Fig. 1A,B). Each MB is composed of four Nb clones (Ito et al., 1997). We used MARCM (Fig. 1C,D) to examine the morphology of one MB Nb clone. After inducing mitotic recombination in a dividing Nb, if the regenerated Nb loses the repressor gene, all subsequently born neurons derived from this repressor-minus Nb are labeled (Fig. 1D). Induction of mitotic recombination in newly hatched larvae (NHL), carrying *GAL4-OK107*, *UAS-mCD8-GFP* and other transgenes required for MARCM, selectively generated MB clones with their entire axon and dendrite projections stained (Fig. 1E). Consistent with the idea that the four MB Nbs produce similar sets of neurons and glial cells (Ito et al., 1997), all marked MB Nb clones were indistinguishable and contained all morphological features characteristic of the whole MB (compare Fig. 1E with 1B).

Dynamic changes in developing MB Nb clones

MB Nbs proliferate throughout the larval and pupal stages. To elucidate how the MBs grow and acquire their characteristic

morphology in adult flies, we followed the development of single MB Nb clones through different stages. Using a pan-neuronal GAL4 line (*GAL4-C155*) and *UAS-mCD8-GFP* in the MARCM system (Lee and Luo, 1999), we generated MB Nb clones in newly hatched larvae (NHL) and examined their morphologies at later developmental stages.

As early as 24 hours after the heat-shock-induced mitotic recombination, marked MB Nb clones have acquired basic projection patterns (data not shown). Following their development through larval life revealed a continuous growth in all MB morphological domains, including cell bodies, calyx, peduncle and two lobes (Fig. 2A-D). These observations indicate that MB Nbs keep dividing throughout larval life and most larval-born MB neurons probably acquire the same basic

morphology; this conclusion is supported by examining 2-cell/single-cell MB clones (see below). In contrast with the five lobes of the adult MB, there are only two lobes in the larval MB, one (the vertical lobe) projecting dorsally and the other (the medial lobe) projecting medially towards the midline. In addition, characteristic side branches (arrows in Fig. 2A-D) form right before bifurcation of the peduncle into lobes and near the end of the medial lobe in the larval MB.

Shortly after puparium formation (APF), dramatic reorganization was observed (Fig. 2E-G). Immediate changes occur in the calyx, which almost completely degenerates by 9 hours APF (arrowhead in Fig. 2F, compared with arrowheads in Fig. 2D,E) and then gradually reappears as a "cotton ball" later (arrowhead in Fig. 2L). The vertical and medial lobes in

Fig. 2. Development of MB neuroblast clones. Composite confocal images reveal the morphology of MB Nb clones, generated in 0-2 hours after larval hatching, at specific developmental stages. (A-D) Four different time points of larva, (E-I) during metamorphosis, and (J-L) the formation of five axonal lobes during pupal stage. MB clones are marked by expression of *mCD8-GFP*. See text for detailed description. Some special features at various developmental stages are pointed, including larva-specific axonal side branches (arrows in A-D), extensive degeneration of larval dendrites right after PF (arrowheads in E and F, compared with arrowhead in D), axonal debris (arrows in G) in the distal regions of larval axonal bundles around 12 hours APF, distinct fascicles of axons in the peduncle around 18 hours APF (arrow in H), stalled axons in the heel region (arrow in I) around 24 hours APF, two distinct dorsal lobes of axons around 36 hours APF (arrows in J), the dendritic processes (arrowheads in K and L), and five adult-specific axonal lobes (arrows in K and L). Note: lower magnification was used to cover the entire clones in E-I due to different orientations of the early pupal clones. The brains in A-F are outlined in gray. Genotype: *GAL4-C155,hs-FLP/Y*(or *y,w*); *FRT^{G13},UAS-mCD8-GFP/FRT^{G13},tubP-GAL80*.
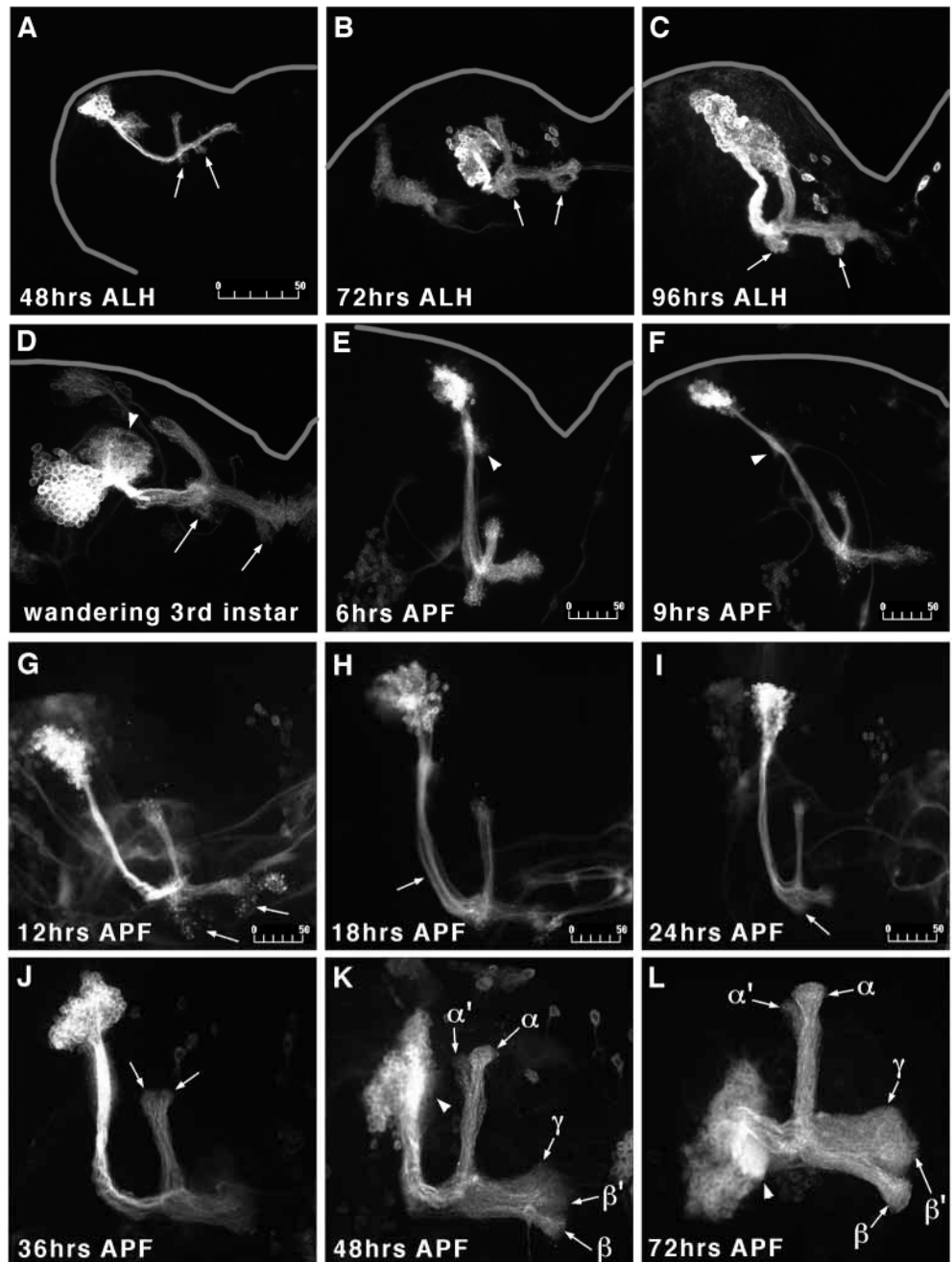
larvae also undergo sequential changes to form five lobes, α and α′ lobes project dorsally while β, β′ and γ lobes project medially (arrows in Fig. 2L). Extensive degeneration with broken debris near the terminals of all axonal bundles is obvious about 12 hours APF (arrows in Fig. 2G). Axonal reorganization is also evident by gradual appearance of multiple distinct axonal fascicles in the peduncle (arrow in Fig. 2H). Around 24 hours APF, numerous axons seem to stall around the end of the peduncle (arrow in Fig. 2I). By 36 hours APF, the dorsally projecting axons appear as two discrete bundles (arrows in Fig. 2J). Later on, dendrites become more and more prominent (arrowhead in Fig. 2K) and all five lobes can be easily detected (arrows in Fig. 2K,L). By 72 hours APF (Fig. 2L), the MB clone looks indistinguishable from the adult clone (compare with Fig. 1E and Fig. 3A). Following development of MB Nb clones through different developmental stages reveals how larval MBs form and evolve into adult MBs. However, the cellular mechanisms underlying these dynamic changes remain to be addressed.

Sequential generation of three distinct types of MB neurons

When mitotic recombination was induced in the developing nervous system, in addition to Nb-derived multicellular clones, marked 2-cell/single-cell clones were observed. A single-cell clone is generated when mitotic recombination is induced in a dividing GMC, while a 2-cell clone is presumably derived from mitotic recombination in a dividing Nb producing a repressor-negative GMC (Fig. 1D). Therefore, neurons appearing as marked single-cell clones are born directly from the mitotic recombination, while neurons in 2-cell clones are born one generation after the mitotic recombination. When the morphologies of 2-cell/single-cell clones, generated after a single pulse of heat shock, were analyzed separately, no difference in projection pattern was detected. Therefore, in our current study, 2-cell and single-cell clones are treated equally as postmitotic neurons born shortly after heat-shock-induced mitotic recombination.

Because of the unique proliferation pattern of MB progenitors (Ito and Hotta, 1992), induction of mitotic recombination in newly hatched larvae (NHL) or late pupae allowed us to generate MB clones selectively. Interestingly, we observed that adult MB neurons in 2-cell/single-cell clones acquired different characteristic axon projections depending on when mitotic recombination was induced (data not shown; see below). Furthermore, all adult MB Nb clones generated in late pupae have only two axon lobes, as compared to five lobes in the Nb clones generated in NHL (data not shown; see below). To elucidate further the cellular components of the MB, we systematically examined the morphologies of marked MB neurons generated by inducing mitotic recombination at different developmental stages. As expected, using the pan-neural *GAL4-C155* in the MARCM, we observed numerous marked non-MB clones when mitotic recombination was induced during most of the larval and early pupal stages (data not shown). In order to facilitate identification of marked MB neurons, the MB neuronal GAL4 line, *GAL4-OK107*, was used in the following studies to restrict labeling largely to MB neurons.

After inducing mitotic recombination in NHL, all marked MB Nb clones contained five axonal lobes when examined in

adult brains (arrows in Fig. 3B). In contrast, inducing mitotic recombination at later developmental stages, we found that four lobes were left in all marked Nb clones generated at the mid-3rd instar stage (arrows in Fig. 3C) and only two lobes remained in Nb clones generated after puparium formation (PF) (arrows in Fig. 3D). In order to determine the identities of various lobes, mosaic brains were immunostained with anti-FasII Ab. As reported previously (Crittenden et al., 1998), FasII is highly expressed in the α and β lobes, weakly expressed in the γ lobe, and not detectable in the α′ and β′ lobes (Fig. 3F and compare Fig. 3E and A). Based on the immunoreactivity for FasII, it appears that the γ lobe is not included in marked MB Nb clones generated at the mid-3rd instar stage (Fig. 3G) and only α and β lobes are present in marked Nb clones generated after PF (Fig. 3H). These results indicate that all neurons projecting into the γ lobe (referred to as γ neurons hereafter) are born before the mid-3rd instar stage and all neurons projecting into the α′ and β′ lobes (referred to as α′/β′ neurons) are born before PF. After PF, all newly born MB neurons (referred to as α/β neurons) project their axons into the α and β lobes.

Examining 2-cell/single-cell clones further substantiated that MB neurons with different axonal projection patterns are born at different developmental stages. When mitotic recombination was induced before the 3rd instar stage, all MB neurons in 2-cell/single-cell clones projected their axons into the γ lobe (Fig. 4A). After inducing mitotic recombination between the mid-3rd instar stage and puparium formation, all 2-cell/single-cell MB clones only consisted of α′/β′ neurons (Fig. 4B). If mitotic recombination was induced after PF, all individually marked MB neurons projected their axons into the α and β lobes (Fig. 4C). Consistent with the observations of the Nb clones, these results indicate that all MB neurons born early in the larval stage are γ neurons, all MB neurons born in the late larva are α′/β′ neurons and all MB neurons born in the pupal stage are α/β neurons.

Sharp switches in the generation of different types of MB neurons

In order to determine the precise timing of these transitions in generating different types of MB neurons, we characterized a large number of 2-cell and single-cell clones generated at various well-defined developmental stages. Newly hatched larvae, less than 2 hours old, were collected and then heat shocked to induce the FLP activity at selected time points. For a given time point, 20 adult brain lobes containing multiple 2-cell/single-cell MB clones were analyzed for the axonal projection patterns of marked neurons (Fig. 4D). All marked MB neurons generated within 2.5 days of incubation at 25°C ALH were found to be γ neurons. The transition from γ neurons to α′/β′ neurons occurred around 3.0 days ALH. When mitotic recombination was induced at 3.0 days ALH, 10% of brain lobes had stopped generating γ neurons and only produced α′/β′ neurons. However, at this time point, the majority of the brain lobes contained mixtures of γ and α′/β′ neurons. The presence of both neuronal types after a single pulse of heat shock probably reflects that the heat-shock period is very close to the transition period. In addition, the perdurance of FLP activity after heat-shock induction may contribute to the generation of mixed population of neurons. These observations suggest that most MB precursors probably stop generating γ

Fig. 3. Lobe compositions of MB Nb clones generated at different stages. Partial composite confocal images of a whole MB (A,E) or MB Nb clones (B-D, F-H) reveal their axonal lobes. Green, GFP fluorescence; Red, FasII immunoreactivity. In the whole MB, GFP fluorescence was detected in all five lobes (A), while the anti-FasII Ab staining was strong in the α/β lobes, weak in the γ lobes, and not detectable in the α'/β' lobes (E). Superimposing the GFP signal with the anti-FasII Ab staining (F-H) reveals the lobe compositions in various MB Nb clones. Note the presence of all five lobes in the MB Nb clone generated in the NHL (B,F); the lack of γ lobe labeling in the clone generated in the mid-3rd instar stage (C,G); and the presence of only the α/β lobes in the clone generated after PF (D,H). Genotypes: (A,E) *UAS-mCD8-GFP/+; GAL4-OK107/+*; (B-D,F-H) *hs-FLP/Y; FRT^{G13},tubP-GAL80/FRT^{G13},UAS-mCD8-GFP; GAL4-OK107/+*.
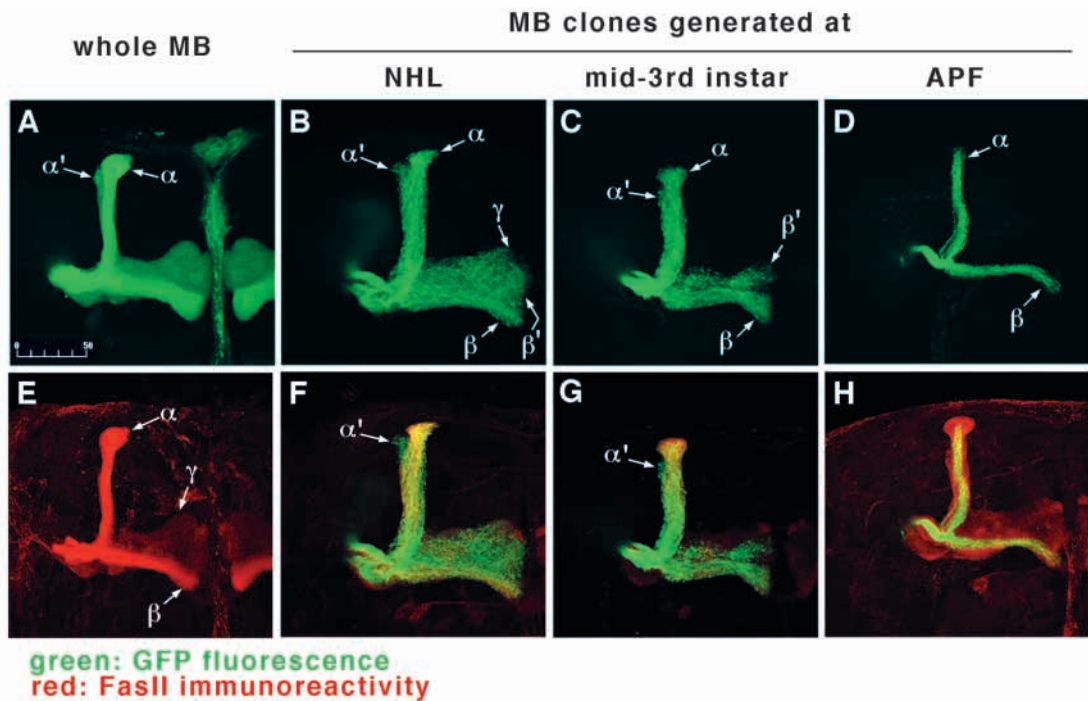

Fig. 4. Sequential generation of three distinct types of MB neurons with characteristic axonal projections. (A-C) Axonal projections of marked MB neurons are revealed by the GFP fluorescence (green) and the MB lobes are immunostained by anti-FasII Ab (red). Inducing mitotic recombination in early larval life results in all GFP-positive axons, derived from isolated MB neurons, projecting into the γ lobe (A). Inducing mitotic recombination in late larval life results in the GFP-positive axons projecting into the FasII-negative α'/β' lobes (B). Inducing mitotic recombination after PF results in GFP-positive axons projecting into the α/β lobes (C). (D) Newly hatched larvae were collected, heat shocked to induce mitotic recombination at various times indicated on the X-axis, and were allowed to resume development at 25°C before the dissection performed within 5 days after eclosion. For a given time point, 20 brain lobes containing multiple 2-cell/single-cell MB clones were examined for the axonal projection patterns of marked MB neurons (A-C). The percentage of brain lobes containing γ, α'/β', and α/β neurons is shown in red, green and blue, respectively, on the Y-axis. Note the ordered generation of morphologically distinct MB neurons. Two sharp transitions occurred at 3 days ALH and around PF. At the transition points, a significant portion of brain lobes contain both types of neurons, which explains why the total percentage of brain lobes containing a certain class of neurons is more than 100%. Genotype: *hs-FLP/Y; FRT^{G13},tubP-GAL80/FRT^{G13},UAS-mCD8-GFP; GAL4-OK107/+*.
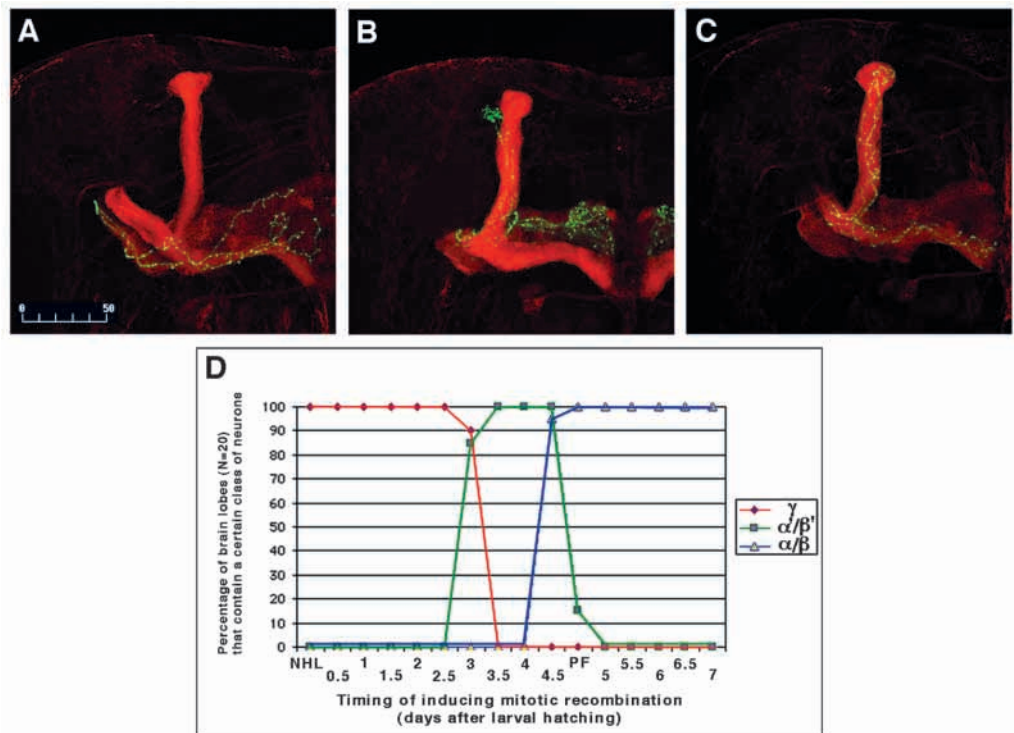
neurons and start producing α′/β′ neurons immediately after 3.0 days ALH, as further supported by the absence of γ neurons resulting from later heat shock. When FLP was induced between 3.5 and 4.5 days ALH, all MB precursors produced α′/β′ neurons.

Again, a sharp transition from α′/β′ to α/β neurons was observed between 4.5 and 5 days ALH. Under our culture condition, most larvae undergo puparium formation between 4.5 and 5 days ALH. To address the timing of the second transition relative to puparium formation, we induced mitotic recombination in newly formed puparia (less than 6 hours after PF), and found that most brain lobes (85%) completely stopped generating α′/β′ neuron shortly after PF. When mitotic recombination was induced after PF (5 days ALH or later), 100% labeled neurons were α/β neurons. Taken together, these data demonstrate that formation of the MB involves sequential and non-overlapping generation of three distinct types of MB neurons from a neuroblast. Furthermore, transitions from γ to α′/β′ neurons and from α′/β′ to α/β neurons occur abruptly and completely at the mid-3rd instar stage and the puparium formation, respectively.

Consistent with previous BrdU labeling experiments (Ito and Hotta, 1992), proliferation of MB Nbs becomes much less active after 8 days ALH, about one day before eclosion. This is supported by the observation that the efficiency of generating marked MB neurons dropped dramatically when mitotic recombination was induced about 8 days ALH or later. If heat shocked around 8 days ALH, about half of the brains (17/36) contained marked MB neurons. When heat shock was around 9 days ALH, marked MB neurons were rarely detected (3/36). In contrast, if heat shocked at earlier time points, almost 100% of brains contained marked MB neurons.

Composition of the adult MB

In order to determine the contributions of different types of neurons to the adult MB, we quantified the total volume of cell bodies present in adult Nb clones having different lobe compositions (see Materials and Methods for details). The average volumes of Nb clones, having all five full lobes, full α′/β′ and α/β lobes, and full α/β lobes alone, were $61,464\pm1,449~\mu m^3$, $39,898\pm4038~\mu m^3$ and $25,802\pm2,308~\mu m^3$, respectively. The difference in volume between 5-lobe clones and 4-lobe clones reflected all γ neurons, born after the NHL stage, that survived into the adult stage. Similarly, the reduction in volume from 4-lobe Nb clones to 2-lobe Nb clones corresponded to all α′/β′ neurons that persisted into the adult stage. Based on this logic, larval-born γ neurons, α′/β′ neurons, and α/β neurons approximately contribute to 35%, 23% and 42% of an adult MB Nb clone, respectively.

Characteristic branching and projection patterns of different MB axons

Examining isolated single-cell clones further reveals detailed morphological features of various types of MB neurons (Fig. 5). Some common features were observed among all MB neurons. In particular, dendritic elaboration superficially looks indistinguishable among various types of MB neurons. Basically, four or so primary dendritic processes branch out from one neuron and each major dendritic process has a claw-like structure usually near its end (arrows in Fig. 5D-F). In addition, all MB processes contain numerous bouton-like

swellings in the region distal to the dendritic-forming segment. Although the three types of neurons share similar dendritic patterns, their axon projection and branching patterns are quite different. The axons of γ neurons remain unbranched until into the γ lobe (Fig. 5A), while the axons of α′/β′ and α/β neurons form two major branches right before entering into the lobe region (Fig. 5B,C). In the γ lobe, several side branches form along the entire process (arrowheads in Fig. 5A). In contrast, in the β′ lobe, side branches always form at characteristic positions, including one side branch in the proximal one quarter region and several side branches in the distal one quarter region (arrowheads in Fig. 5B). In other lobes, fewer or no significant side branches were observed. Interestingly, we noted that some axons in the α and β lobes made several helical turns along their paths (Fig. 4C), similar to certain Golgi-stained housefly MB neurons (Strausfeld, 1976). Systematic analysis of more isolated single-cell clones may allow one to identify subtypes of MB neurons based on subtle morphological features.

Contrasting behaviors of γ and α′/β′ neurons during metamorphosis

As described earlier, a larval MB Nb clone has only two axonal lobes and both lobes grow simultaneously at all stages examined (Fig. 2A-D), suggesting that most MB neurons probably have their axons branched into both lobes in larvae. Our study further demonstrate that γ and α′/β′ neurons are born sequentially in the larva and γ neurons only project their axons medially into the γ lobe in adults. This raises several interesting questions about how the MB is reorganized during metamorphosis. For instance, it remains unclear whether late-born neurons (α′/β′ neurons) complete their morphogenesis before PF and how early-born neurons (γ neurons) reorganize their axonal projections during metamorphosis.

To observe the detailed morphologies of larval MB neurons and their morphological changes during metamorphosis, we examined individually marked neurons, born during different larval stages, before and after PF. Mitotic recombination was induced in NHL, 2-day-old larvae, and 3.5-day-old larvae, in order to generate early-born γ neurons, late-born γ neurons and α′/β′ neurons, respectively (Fig. 4D). Although *GAL4-OK107* appears to drive expression of the *UAS-mCD8-GFP* in most, if not all, adult MB neuron (see Discussion), the *GAL4-OK107*-dependent expression might not be as strong in all larval MB neurons. Therefore, another MB-enhancer trap GAL4 line, *GAL4-201Y* (Yang et al., 1995), was used in parallel for collecting more γ neurons, and consistent results were obtained.

Examining the morphologies of isolated 2-cell and single-cell clones revealed that all larva-born MB neurons (n=100) acquired similar morphological features before PF. Both γ and α′/β′ neurons have two major axonal branches, one projecting dorsally and the other projecting medially (Fig. 6A-C). In addition, consistent with the morphologies of larval Nb clones, two characteristic side branches, one slightly proximal to the site of axonal bifurcation and the other around the distal one-third position of the medial axonal process (arrowheads in Fig. 6A-C), are present in every larval-born MB neuron examined. No detectable difference was observed between early-born (n=40) and late-born (n=20) γ neurons. However, some subtle differences were observed between γ (n=60) and α′/β′ (n=40)

Fig. 5. Morphological characterization of three types of MB neurons. (A-C) Composite confocal images of isolated adult MB neurons reveal their characteristic morphologies at single-cell level. These marked MB neurons were born during the early larval (A), late larval (B) and pupal stage (C), respectively. Arrowheads point at axonal side branches (A,B). Note the characteristic axonal side branches in the α'/β' neuron (arrowheads). (D-F) High magnification of the dendritic regions of A-C reveals claw-like structures near the ends of major dendritic branches (arrows). Genotype: *hs-FLP/Y; FRT^{G13},tubP-GAL80/FRT^{G13},UAS-mCD8-GFP; GAL4-OK107/+.*
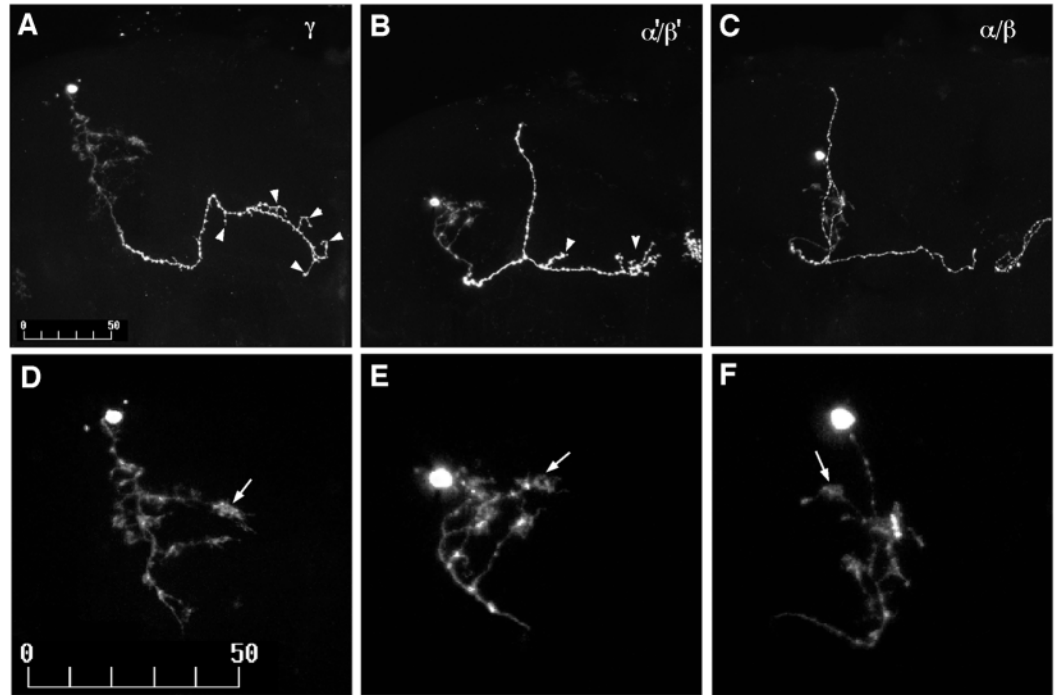
neurons before PF. In contrast to γ neurons, although α'/β' neurons acquire the basic morphological features within 24 hours, their axonal side branches are quite short and their dendritic trees look relatively immature (arrows in Fig. 6A-C), as evidenced by the absence of claw-like structures found in γ neuron dendrites.

Interestingly, shortly after PF, γ and α'/β' neurons undergo different reorganizations (compare Fig. 6F with D,E). Extensive degeneration of dendrites was observed in all γ

neurons when examined around 18 hours APF (n=60), while relatively mild shorting happened to the dendrites of α'/β' neurons (n=50) (arrows in Fig. 6D-F). In addition, degeneration of axonal branches was noted in all γ neurons (n=60). However, the main processes in the peduncle remained relatively intact (Fig. 6D,E). Later, around 24 hours after PF, these truncated processes of γ neurons re-extended medially to form the γ lobe (Fig. 7D) such that γ neurons changed their axonal projection pattern during metamorphosis. This dynamic

Fig. 6. Different behaviors of γ and α'/β' neurons during metamorphosis. 2-cell/single-cell MB clones were generated by inducing mitotic recombination during the 1st instar stage (A,D), the early 3rd instar stage (B,E) or the late 3rd instar stage (C,F). Their morphologies were examined right before PF (A-C) or about 18 hours after PF (D-F). Note that all larval-born MB neurons share basic morphological features, including the axonal projection pattern and characteristic axonal side branches (arrowheads in A-C), before metamorphosis. After puparium formation, extensive degeneration of axonal branches in the lobe region is evident in γ neurons, while α'/β' neurons extend fine processes (arrowheads in F) near the axonal terminals. In addition, dendritic processes (arrows) degenerate almost completely in γ neurons but only partially in α'/β' neurons during early metamorphosis. Genotype: (A,B,D,E) *hs-FLP/Y; FRT^{G13},tubP-GAL80/FRT^{G13},UAS-mCD8-GFP,GAL4-201Y* and (C,F) *hs-FLP/Y; FRT^{G13},tubP-GAL80/FRT^{G13},UAS-mCD8-GFP; GAL4-OK107/+.*
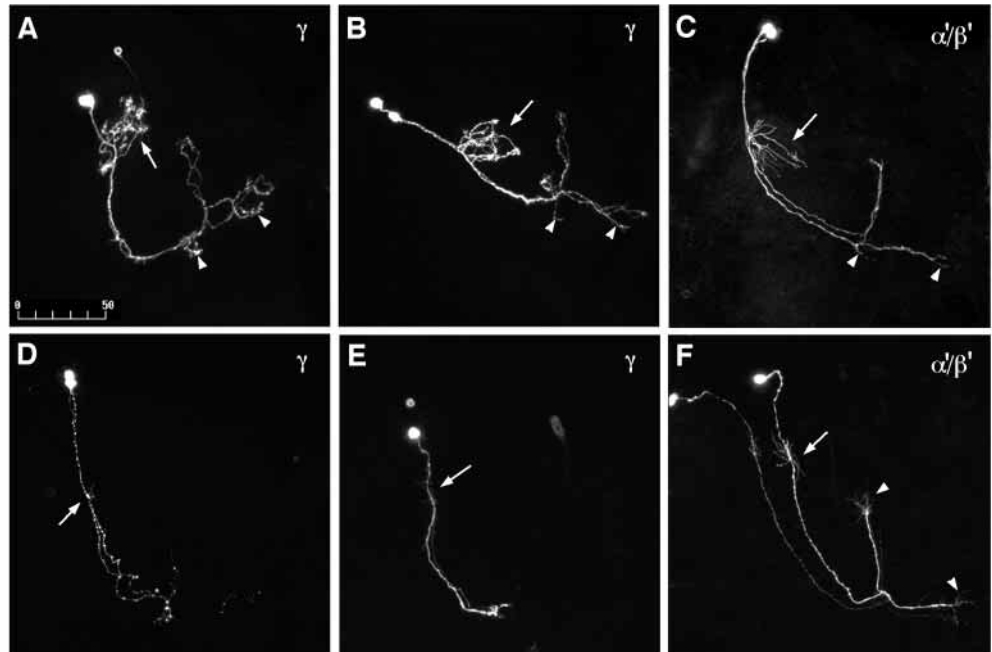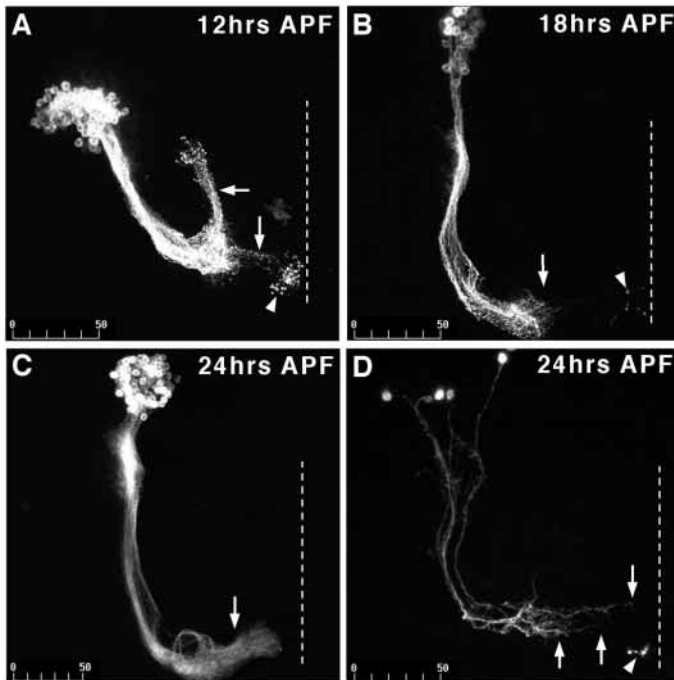
Fig. 7. Axon reorganization of γ neurons during metamorphosis.
(A-C) Selectively marked γ neurons in MB Nb clones were examined
12, 18 and 24 hours after PF. Degeneration of both larval axon lobes
occurs shortly after PF (arrows in A). Degradation of γ axons is
largely limited to the lobes, as axons appear intact through the end of
the peduncle (arrow in B). At 24 hours APF, γ axons are in the
process of re-extending medially to form the adult γ lobe (arrow in
C). (D) Around 24 hours APF, isolated γ neurons were in the process
of extending their axons into the γ lobe, as suggested by various
ending positions of axons (arrows). All clones were generated in
NHL. Arrowheads indicate axon debris and broken lines represent
the midline. Genotype: *hs-FLP/Y; FRT^{G13},tubP-
GAL80/FRT^{G13},UAS-mCD8-GFP,GAL4-201Y.*

reorganization of γ axons during early metamorphosis was
clearly revealed, when using *GAL4-201Y* to selectively label γ
neurons in Nb clones (Fig. 7A-C). By 36 hours after PF,
growing γ axons appeared fully extended (data not shown). In
contrast, in the α′/β′ neurons, only the larva-specific axonal
side branches degenerated when 2-cell/single-cell clones were
examined between 12 and 24 hours APF (*n*=50). Instead, active
extension of fine branches was observed at the axonal terminals
of α′/β′ neurons during the early pupal stage (arrowheads in
Fig. 6F). Therefore, no dramatic change occurs in major axonal
branches of α′/β′ neurons during metamorphosis, which
explains why incomplete degeneration was observed in the
larval lobes of Nb clones by 12 hours after PF (Fig. 2G).

DISCUSSION

Summary of mushroom body development

The demonstrations of sequential generation of three types of
MB neurons and differential metamorphosis of γ and α′/β′
neurons provide a cellular basis for development of the
mushroom bodies (Fig. 8). Taken together with previous
studies, neurogenesis of one MB involves continuous

proliferation of four equivalent Nbs through the embryonic,
larval and pupal stages (Ito et al., 1997). During
embryogenesis, four MB Nbs generate less than 50 neurons,
which probably project their axons exclusively into the γ lobe
of adult flies (Armstrong et al., 1998). After larval hatching,
one MB Nb produces about 500 more neurons. Based on their
axonal projection patterns, there are three types of MB
neurons. These three stereotypic types of axonal projections
lead to formation of five distinct axonal lobes in the adult MB.
Before the mid-3rd instar stage, all MB precursors generate γ
neurons (red in Fig. 8). Between the mid-3rd instar stage and
the PF, all MB precursors generate α′/β′ neurons (green in Fig.
8). Both γ and α′/β′ neurons acquire similar projection patterns
before PF. After PF, γ neurons undergo dramatic
reorganizations in order to change the axonal projection
pattern, while α′/β′ neurons remain relatively unchanged.
Meanwhile, all newly born MB neurons after PF become α/β
neurons (blue in Fig. 8), whose axonal processes form the
adult-specific α/β lobes.

Studying wild-type neurogenesis with the MARCM system

Because of a central role for the MBs in *Drosophila* learning
and memory, development and cellular organization of the
MBs have been investigated by various approaches. Using the
MARCM, we were able to mark the entire morphology of a
subset of MB neurons, based on their dates of birth. The GAL4
activity is essential for labeling the repressor-lacking
homozygous cells in the MARCM. Three lines of evidence
suggest that both *GAL4-C155* and *GAL4-OK107*, used for most
of these studies, label almost all MB neurons in adult. First,
both lines allow us to visualize all MB lobes. Second, the
ability to label various subsets of MB neurons generated at
different stages supports ubiquitous expression of the GAL4s
in adult MB neurons. Third, one MB Nb clone, generated in
NHL using MARCM with both *GAL4-C155* and *GAL4-
OK107*, is estimated to contain about 500 cell bodies (based
on cell counting of Nb clones through serial confocal sections),
which is roughly equivalent to one quarter of axons passing
through the peduncle (Technau and Heisenberg, 1982).
However, there may be quantitative differences in expression
levels. For instance, the *GAL4-C155*-driven expression is
weaker in adult γ and α′/β′ neurons (data not shown). In
addition, the MB expression patterns of both GAL4 lines at
different developmental stages remain to be examined.
Therefore, we can not rule out the possibility that *GAL4-C155*
or *GAL4-OK107* has no detectable expression in a very small
subset of MB neurons, especially when examining clones at
larval and pupal stages.

Another key experimental manipulation in this study is the
temporal control of mitotic recombination, which is achieved
by heat-shock-induced expression of the FLP recombinase.
Specificity of this temporal control is fully supported by the
stage-specific patterns of marked clones. However, perdurance
of the FLP activity should be considered when determining the
timing of cell types switching. At the transition stages, we
found that two distinct types of marked MB neurons are
simultaneously generated in most brain lobes (Fig. 5D). The
most-likely explanation is that the cell fate transition occurs so
abruptly that the prolonged FLP activity following heat shock
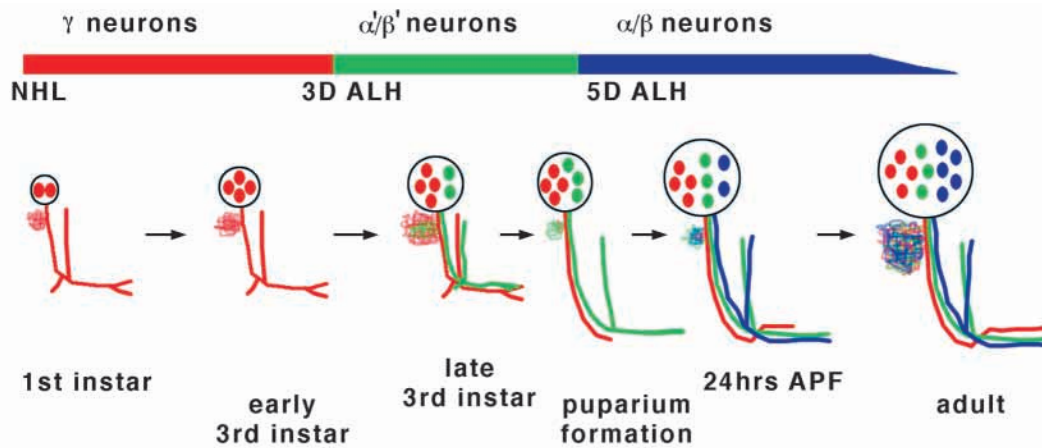induces mitotic recombination both before and after the

Fig. 8. Summary of the mushroom body development. One MB is derived from four identical Nbs in each hemisphere. By asymmetric divisions through the embryonic, larval and pupal stages, each MB Nb generates about 500 neurons (Technau and Heisenberg, 1982), which can be classified into three types based on their axonal projection patterns. These three types of MB neurons are born in a specific temporal order. γ neurons (red) are born before the mid-3rd instar stage, α′/β′ neurons (green) are born between the mid-3rd instar stage and PF, and α/β neurons (blue) are born after PF. In the larval life, all MB neurons, including γ and α′/β′ neurons, acquire similar morphologies, although both dendritic elaboration and axonal side branching look relatively immature in α′/β′ neurons. During the early pupal stage, γ neurons change their axonal projection pattern after partial degeneration of their axons. In addition, degeneration of larval dendrites followed by elaboration of adult dendrites occurs in pupae. The axons from different types of neurons are fasciculated into separate bundles. In the lobe region, the axonal bundles from γ neurons, α′/β′ neurons and α/β neurons correspond to the γ lobe, the α′/β′ lobes and the α/β lobes, respectively.

switching in cell fate. Because of the difficulty in evaluating perdurance of the FLP activity, the timing of heat shock coincides more accurately with the end rather than the beginning in generating a certain type of MB neurons.

Regulation of neuronal fates during development

How a small number of neuronal progenitors generate a large number of different types of neurons is a general question in developmental neurobiology. The fact that three different types of MB neurons are born from a single Nb in a non-overlapping temporal order suggests several possible underlying mechanisms. One possibility is that a completely intrinsic program operates in the Nb for switching its cell fate in an age-dependent manner. Alternatively, the Nb may change its cell fate in response to specific environmental cues. Other possibilities include cell-cell interactions among postmitotic neurons or differentiation of postmitotic neurons according to other environmental variables. Interestingly, the second switch in the cell fates of MB neurons precisely coincides with puparium formation, suggesting that the insect steroid hormone, ecdysone, may mediate this cell fate change. Consistent with this idea, the first switch in the MB neuronal cell fates occurs in the mid-3rd instar stage and roughly coincides with an ecdysone peak known to be important in preparation for puparium formation (Fisk and Thummel, 1998). Ecdysone has been well documented in regulating various aspects of insect development by mediating different stage-specific and tissue-specific transcriptional responses (for review, see Thummel, 1996). During metamorphosis, it is known that ecdysone activity is required for reorganization of the nervous system, including both degeneration of larval-specific neurites and formation of adult-specific neurites (Schubiger et al., 1998). However, it has not yet been reported that ecdysone plays a direct role in regulating neuronal cell fates. It will be interesting to test the role of ecdysone in cell

fate switching of MB neurons by manipulating the activities of ecdysone receptors with MARCM.

The ordered appearance of distinct neuronal cell types has been a general phenomenon in the developing nervous system. Comprehensive studies on embryonic neurogenesis of the *Drosophila* ventral nerve cord have defined individual Nbs and their characteristic cell lineages (for review, see Goodman and Doe, 1993; Doe and Technau, 1993). Similar phenomenon was observed in higher organisms. Lineage analysis using retroviral vectors reveals the presence of multipotential progenitors during development of various parts of vertebrate nervous systems (for review, see Cepko et al., 1998). Both in vivo and in vitro studies suggest that the differentiation of multipotential neural progenitor cells into specific classes of postmitotic neurons or glial cells occurs gradually and involves a progressive restriction in the range of fates available to individual cells (for review, see Edlund and Jessell, 1999). Most of these developmental processes are far more complicated than neurogenesis of MB neurons. For instance, development of the vertebrate retina involves a heterogeneous population of progenitors, simultaneous generation of two or more types of cells and gradual transitions among different types of cells (for review, see Cepko et al., 1996). These complexities make mechanistic studies on the cell fate regulation of vertebrate neural progenitors more challenging. Elucidating the molecular mechanisms for the ordered generation of distinct types of MB neurons may shed light on understanding similar developmental processes in higher organisms.

Possible functions of different types of MB neurons

Our studies provide new insights into the function of three distinct classes of neurons. Since the α/β neurons are born starting from puparium formation, they obviously play adult specific roles. It is interesting that they are among the largest

group of the neurons from our estimation of the volume contribution (42%) and their axons are the most densely packed. The γ neurons, on the contrary, probably play important roles in larval stages since they are likely born in embryos (Tettamanti et al., 1997) and certainly early stages of larval life (this study). By the end of the third instar larva, the dendrites and axon projections into the lobes are highly elaborate (Fig. 7A,B). However, the γ neurons undergo the most dramatic changes in the first day of pupa life so that their dendrites as well as their axon branches into the larval vertical and medial lobes are completely degenerated. Curiously, the adult projection pattern is medial lobe-specific, suggesting that the γ neurons may change their network property altogether in larval and adult life.

The α'/β' neurons, although born in late stage of larva life, may not be fully differentiated at the end of larval life judging from the immature dendrites compared to the γ neurons at the end of third instar (Fig. 7C, compared to Fig. 7A,B), so their functions may also be largely adult-specific. However, they may play important roles in the transition between the larval mushroom body and adult mushroom body both from the point of axon guidance and dendritic morphogenesis, and from the point of maintaining connections with input and output neurons. Since the γ neurons completely change connection pattern during metamorphosis, the α'/β' neurons, whose axon and dendrite projections may follow the routes established by the γ neuron in larva, may be instrumental to ensure that the adult-specific α/β neurons project their axons correctly into both medial and dorsal lobes, and their dendrites to occupy the calyx region. Their function in axon guidance may be analogous to that of the subplate neurons in axon guidance of mammalian cerebral cortical neurons (McConnell et al., 1989; Ghosh et al., 1990). Unlike the subplate neurons, the α'/β' neurons are themselves not pioneer neurons. Their roles may be to transfer the routes established by the pioneering γ neurons to the later-born α/β neurons.

It has been shown that *Drosophila* larvae are capable of olfactory learning (Aceves-Pina and Quinn, 1979) and memory can persist through metamorphosis (Tully et al., 1994). Although it has not been formally demonstrated that larval mushroom bodies are essential for larval learning, it is quite likely that they do play a role judging from the conserved organization of the MB neurons between larva and adult. For instance, both larval and adult mushroom bodies contain the calyx in analogous positions, and the axon projections are consisted of the dorsal and the medial lobes and in conserved relative positions. Moreover, many genes that are preferentially expressed in adult mushroom bodies are also preferentially expressed in larval mushroom bodies, including genes that are implicated in olfactory learning and memory (Crittenden et al., 1998). In light of our findings, α'/β' neurons may play important roles in connecting the larval and adult functions of the mushroom bodies. It will be interesting to determine whether larvae younger than third instar, before the birth of the α'/β' neurons, are capable of olfactory learning and their memory can persist through metamorphosis.

In conclusion, using clonal analysis made possible by the MARCM system, we have illustrated here the sequential generation of three distinct types of mushroom body neurons from a single neuroblast. We further described the projection of axons and dendrites of these neurons at single cell resolution, and their behavior during metamorphosis. These studies will now enable us to study the molecular mechanisms of these developmental events using genetic mosaic analysis (Lee and Luo, 1999), which will eventually contribute to greater understanding of the function of the MB neurons in the neuronal network that is essential for insect learning and memory.

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