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Embryonic and larval development of the *Drosophila* mushroom bodies: concentric layer subdivisions and the role of *fasciclin II*

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SUMMARY

Mushroom bodies (MBs) are the centers for olfactory associative learning and elementary cognitive functions in the arthropod brain. In order to understand the cellular and genetic processes that control the early development of MBs, we have performed high-resolution neuroanatomical studies of the embryonic and post-embryonic development of the *Drosophila* MBs. In the mid to late embryonic stages, the pioneer MB tracts extend along Fasciclin II (FAS II)-expressing cells to form the primordia for the peduncle and the medial lobe. As development proceeds, the axonal projections of the larval MBs are organized in layers surrounding a characteristic core, which harbors bundles of actin filaments. Mosaic analyses reveal sequential generation of the MB layers, in which newly produced Kenyon cells project into the core to shift to more distal layers as they undergo further differentiation. Whereas the initial extension of the embryonic MB tracts is intact, loss-of-function mutations of *fas II* causes abnormal formation of the larval lobes. Mosaic studies demonstrate that FAS II is intrinsically required for the formation of the coherent organization of the internal MB fascicles. Furthermore, we show that ectopic expression of FAS II in the developing MBs results in severe lobe defects, in which internal layers also are disrupted. These results uncover unexpected internal complexity of the larval MBs and demonstrate unique aspects of neural generation and axonal sorting processes during the development of the complex brain centers in the fruit fly brain.

Key words: Neurogenesis, Pathfinding, Learning, Memory, *dunce*, Actin filaments, *Drosophila*

INTRODUCTION

Mushroom bodies (MBs) of the arthropod brain are a pair of prominent neuropil structures whose internal and external connections are highly conserved across species (Strausfeld et al., 1998). Behavioral studies imply that MBs are centers for higher-order functions, including olfactory learning (Davis, 1996; Heisenberg, 1998), courtship behavior (Ferveur et al., 1995; O'Dell et al., 1995; McBride et al., 1999) and elementary cognitive functions, such as visual context generalization (Liu et al., 1999).

In the adult *Drosophila* brain, each of the MBs comprises a large number of densely packed parallel fibers (Fig. 1), which are systematically organized into distinct computational networks (Yang et al., 1995; Ito et al., 1998; Strausfeld et al., 1998). The MB cell bodies (Kenyon cells) are located at the dorsal cortex, extending their dendrites into the calyx, which receives olfactory information from the antennal lobes via the prominent inner antennocerebral tract (iACT). More distally, MB axons constitute a massive parallel tract called the peduncle, which splits at its distal tip into two main branches: one projecting dorsally and the other medially. The dorsal branch is composed of two inter-wined lobes, α and α′, and the medial lobe is composed of three parallel lobes, β, β′ and γ (Crittenden et al., 1998).

Developmental studies have shown that Kenyon cells are produced by the division of the four MB neuroblasts, which are born at an early embryonic stage (Noveen et al., 2000) and divide continuously throughout development (Truman and Bate, 1988; Prokop and Technau, 1991; Ito and Hotta, 1992; Prokop and Technau, 1994; Ito et al., 1997). Systematic clonal analysis has demonstrated that a single MB neuroblast sequentially generates three types of distinct MB neurons (Lee et al., 1999). Neurons that project into the γ lobe of the adult MBs are born first, before the mid third instar larval stage. Subsequently, neurons projecting into the α′ and β′ lobes are produced at the late third instar stage, and neurons projecting into the α and β lobes are born in the pupal period. Whereas all the larval MB neurons bifurcate into the dorsal and medial lobes, most of the larval neural projections degenerate to be reorganized into the adult structure during the pupal period.

Molecular genetic analyses (Kurusu et al., 2000; Martini et al., 2000; Noveen et al., 2000; Callaerts et al., 2001) have revealed that a set of nuclear regulatory genes, *eyeless* (*ey*), *twin of eyeless* and *dachshund* (*dac*), which are implicated in

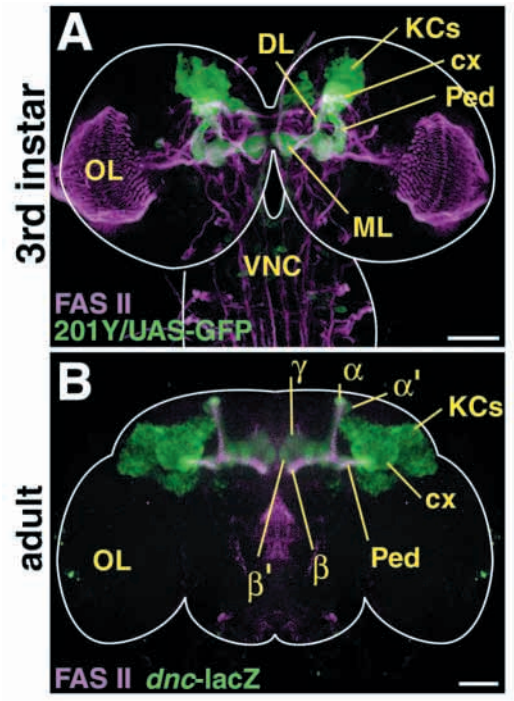


Fig. 1. The larval and adult mushroom bodies. (A) Third instar larval brain stained with anti-FAS II (magenta) and GFP (green) driven by a MB *GAL4* line, 201Y. (B) Adult brain stained for FAS II (magenta) and *dnc-lacZ* (green). α and α′, adult dorsal lobes; β, β′ and γ, adult medial lobes; cx, calyx; DL, dorsal lobe; KCs, Kenyon cells; ML, medial lobe; OL, optic lobe; Ped, peduncle; VNC, ventral nerve cord. Reconstruction of optical sections. Scale bars: 50 μm.

eye development, also have important functions in MB development. Mutations of *ey* completely disrupt the neuropil structures of the MBs and a null mutation of *dac* results in marked disruption and aberrant projections of MB axons. Furthermore, expression of *ey* and *dac* are independently regulated in the course of MB development, arguing for a distinct combinatorial code of developmental regulatory genes as compared with eye development.

We have performed high-resolution neuroanatomical studies of the embryonic and post-embryonic MBs of the *Drosophila* brain using various axonal- and cell type-specific markers. We first describe the initial stages of MB tract development in the embryonic protocerebral anlage. We then describe formation of discrete concentric layers in the larval peduncle and lobes as topological projections of concentric distoproximal subdivisions of the Kenyon cells. FAS II (Fas2 – FlyBase) is expressed at high level in the inner and outer layers but is absent in the central core, which is instead constituted of densely packed newly born fibers rich in actin filaments. Mutational analyses show that the normal development of the lobes and the underlying layer structure require *fas II* (*Fas2* – FlyBase) function. Furthermore, ectopic overexpression of FAS II in the developing MBs caused severe alterations of the branching patterns of the medial and dorsal lobes. These results uncover unexpected internal complexity of the larval MBs, and demonstrate unique aspects of neural generation and axonal sorting processes during the development of the complex brain centers in the fruit fly brain.

MATERIALS AND METHODS

Drosophila stocks

The following fly strains were used: wild-type (Oregon-R), MB-*GAL4* enhancer-trap lines (201Y, 238Y, c739) (Yang et al., 1995), *GAL4-OK107* enhancer trap (Connolly et al., 1996), *elav*^{C155}-GAL4 (Lin et al., 1994), UAS-*tau-lacZ* (Callahan and Thomas, 1994), UAS-*fas II* [PEST (–)] (Lin et al., 1994), *UAS-GFP* (provided by E. Hafen, Zurich) and *UAS-mCD8::GFP* (Lee and Luo, 1999). P[*dnc-lacZ*] flies, which express *lacZ* driven by the *dunce* (*dnc*) enhancer (Qiu and Davis, 1993), were provided by R. Davis (Houston, TX). *fas II*^{e86} (hypomorphic, 50%), *fas II*^{e76} (hypomorphic, 10%) and *fas II*^{eB112} (null allele) have been described by Grenningloh et al. (Grenningloh et al., 1991). Stocks used for ectopic *fas II* expression were *UAS-fas II[PEST(–)]/CyO* and *UAS-mCD8::GFP/FM7c; UAS-fas II[PEST(–)]/CyO*.

MARCM mosaic analysis

The following fly stocks were used: GAL4^{c155} hs-FLP; FRT^{G13} UAS-mCD8::GFP/CyO and FRT^{G13} tubP-GAL80/CyO for layer development analyses, and hs-FLP tubP-GAL80 FRT19A; 201Y/SM1 and fas II^{eB112} FRT19A/FM7c; UAS-GFP-T2/SM1 for fas II mutant analyses. Mosaic clones were generated as described (Lee and Luo, 1999). Egg (0- to 1.5-hour-old) collection was done for 1.5 hours on standard food at 25°C. A single 60 minute heat shock at 37.5°C was applied at 27 to 28 hours after egg laying for induction of mitotic recombination in the first instar stage, and 75 to 76 hours after egg laying for that in the early third instar stage. Clones were examined at the wandering larval stage.

Immunocytochemistry and confocal microscopy

Immunostaining of brains was as described previously (Hirth et al., 1995; Tettamanti et al., 1997; Nagao et al., 2000; Kurusu et al., 2000). The following primary antibodies were used: goat FITC-conjugated anti-horseradish peroxidase (HRP) diluted 1:300 (Jackson ImmunoResearch); Alexa Fluor-conjugated phalloidin diluted 1:40 (Molecular Probes); rabbit anti-β-Gal diluted 1:1000 (Chemicon International); rabbit anti-EY diluted 1:300 (gift from U. Walldorf); mouse anti-DAC (mABdac2-3) diluted 1:250; mouse anti-FAS II (mAB1D4) (Grenningloh et al., 1991) diluted 1:5; rabbit anti-DIF (Cantera et al., 1999) diluted 1:250; rabbit anti-Synaptotagmin (Littleton et al., 1993) diluted 1:1500 and rat anti-mCD8α diluted 1:100 (Caltag). FITC-, Cy3- or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch) were used at dilution of 1:400. Confocal images were captured with a Zeiss LSM410 or LSM510 confocal microscope. Optical sections were 1-4 μm. Images were processed digitally and then arranged with Adobe PhotoShop.

RESULTS

Pioneering the embryonic axonal tracts

Previous studies have shown that the orthogonal organization of the larval MBs emerges during mid to late embryonic stages (Tettamanti et al., 1997; Noveen et al., 2000). In order to gain insights on the cellular and genetic mechanisms involved in the very early stages of MB development, we carried out confocal examinations of the embryonic MBs using specific markers in conjunction with an anti-FAS II antibody, which labels the major pioneer tracts of the developing brain (Nassif et al., 1998; Nagao et al., 2000).

The reporter gene expression of a *GAL4* line 238Y becomes prominent by embryonic stage 14 in a subset of the four MB neuroblasts and their progenies, which locate at the most anterior region of the brain in neuraxis (Fig. 2A) (Tettamanti

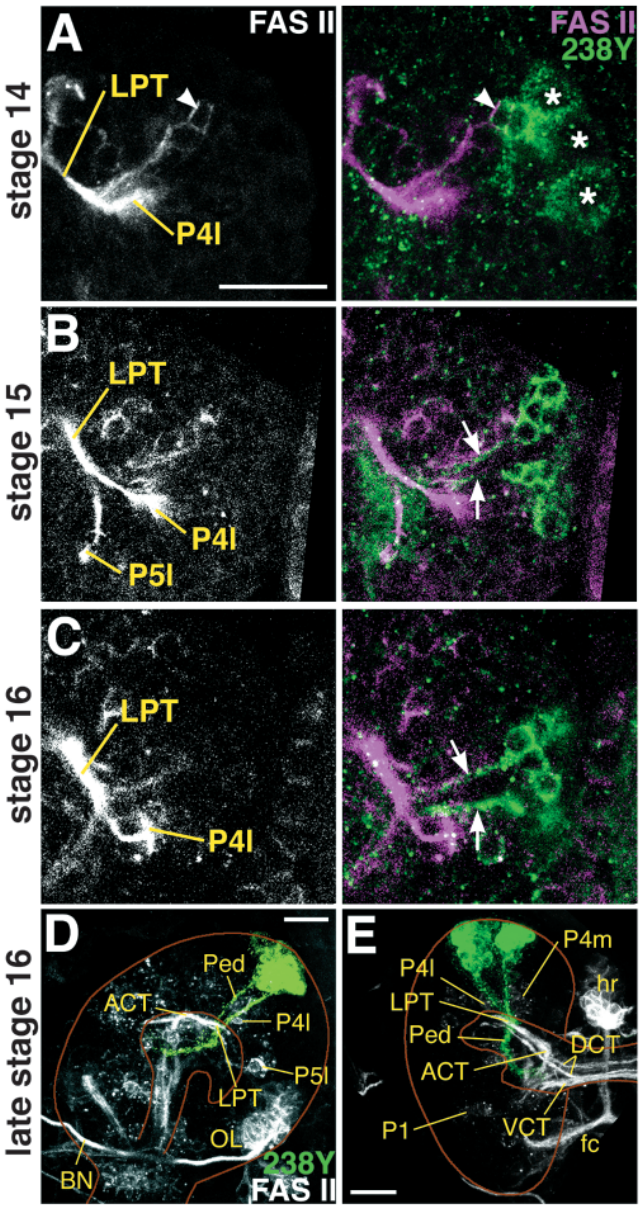


Fig. 2. Embryonic development of the mushroom body (MB) axonal tracts. (A-C) Single optical sections showing the embryonic MB tracts. Lateral views. The embryonic MB tracts are labeled with *UAS-tau-lacZ* (green) driven by 238Y-*GAL4*. The major brain tracts are visualized with anti-FAS II antibody (magenta). (A) Stage 14. Arrowheads indicate an interface cell that co-expresses FAS II and 238Y on its surface. MB neuroblasts are indicated with stars. (B) Stage 15. The 238Y MB neurons extend thin pioneer axons (arrows) along the FAS II-expressing cells. (C) Stage 16. The MB tracts converge at LPT. FAS II expression on the nearby cells is down regulated by this stage while the growing MB tracts (arrows) become more prominent. (D,E) Late stage 16. Reconstructed from optical sections. Lateral (D) and dorsal (E) views. The 238Y signal (green) is selectively enhanced to show the embryonic MBs. Major FAS II tracts also are shown (white). The embryonic brain hemisphere and its core neuropil are outlined with brown line. ACT, antennocerebral tract; BN, Bolwig's nerve; DCT, dorsal commissural tract; fc, frontal commissure; LPT, lateral protocerebral tract; MPT, medial protocerebral tract; OL, optic lobe; P1, P4l, P4m, P5l, fiber tract founder clusters (Nassif et al., 1998) Ped, peduncle; VCT, ventral commissural tract. Scale bars: in A, 10 μm for A-C; 10 μm in D,E.

et al., 1997). Concomitant with this, a group of FAS II-positive cells was observed near the axonal tract founder cluster P4l. As axogenesis of the embryonic Kenyon cells started at late embryonic stage 14, the pioneer MB axons (arrows in Fig. 2B) extended along the FAS II-positive cells towards the prominent lateral protocerebral tract (LPT). By stage 16, as the axonal outgrowth further proceeded, additional MB fibers joined the initial tract by selective fasciculation while FAS II expression on the nearby cells started fading away. The two closely apposed sets of the axonal projections (arrows in Fig. 2C) converged at the LTP.

By late stage 16, the MB tracts further extended anteriorly beyond the LTP to form the distal part of the embryonic peduncle (Fig. 2D,E). They made a sharp medial turn closely at the border of the embryonic proto- and deutocerebrum, giving rise to the embryonic medial lobe. A prominent tract connecting the MB primordium with the deutocerebral cell cluster was also established by this stage (ACT in Fig. 2E). By late stage 17, the dorsal lobe emerged as a collateral outgrowth at around the turn, the MB axons started to express FAS II and calyces emerged only near the end of embryogenesis (data not shown) (Tettamanti et al., 1997; Noveen et al., 2000).

Layer development in the larval mushroom bodies

While the four MB neuroblasts continued dividing up to the late pupal stage supplying increasing numbers of Kenyon cells (Truman and Bate, 1988; Ito and Hotta, 1992), the newly formed larval MB axons followed the medial and the dorsal lobe projections that were pioneered at the embryonic stage with a concomitant increase in the sizes of the lobes. By contrast, a set of genes was turned on in the Kenyon cells after the hatching of the first instar larvae in slightly different patterns in both the cell bodies and their projections (Fig. 3). As development proceeded, these differential gene expression patterns became more evident in the second instar larval stage (Fig. 4). While the DAC protein is expressed in most of the Kenyon cells (Kurusu et al., 2000; Martini et al., 2000; Noveen et al., 2000), *dnc-lacZ* was expressed in a small subset of cells peripherally positioned in each of the Kenyon cell clusters originated by the four MB neuroblasts (Fig. 4A; see Fig. 10B for schematic presentation). Expression of 201Y was detected in another subset of cells located more centrally in each of the Kenyon cell clusters, whereas c739 was widely expressed in most of the Kenyon cells.

Remarkably, these differential expression patterns observed in the Kenyon cells were topologically reflected in their axonal projections in the peduncle and lobes (Fig. 4D-L): *dnc-lacZ* was detected in the outermost surface layer of the peduncle and lobes; 201Y was detected in both the surface and middle layers; and c739 was detected in most axons, a pattern similar to that of FAS II.

As development further proceeded, further subdivisions emerged in the third instar larval stage with increasing numbers of Kenyon cells and their axons (Fig. 5). Moreover the expression patterns of the 201Y and c739 markers changed in both cell bodies and their projections; 201Y was then detected in many on of the Kenyon cells (Fig. 5B) and their projections (Fig. 5E,H) obscuring its peripheral pattern in the previous larval instar; c739 was then detected in a group of cells located near each of the neuroblasts (Fig. 5C). The axons of the c739-expressing cells projected into an inner layers of the peduncle

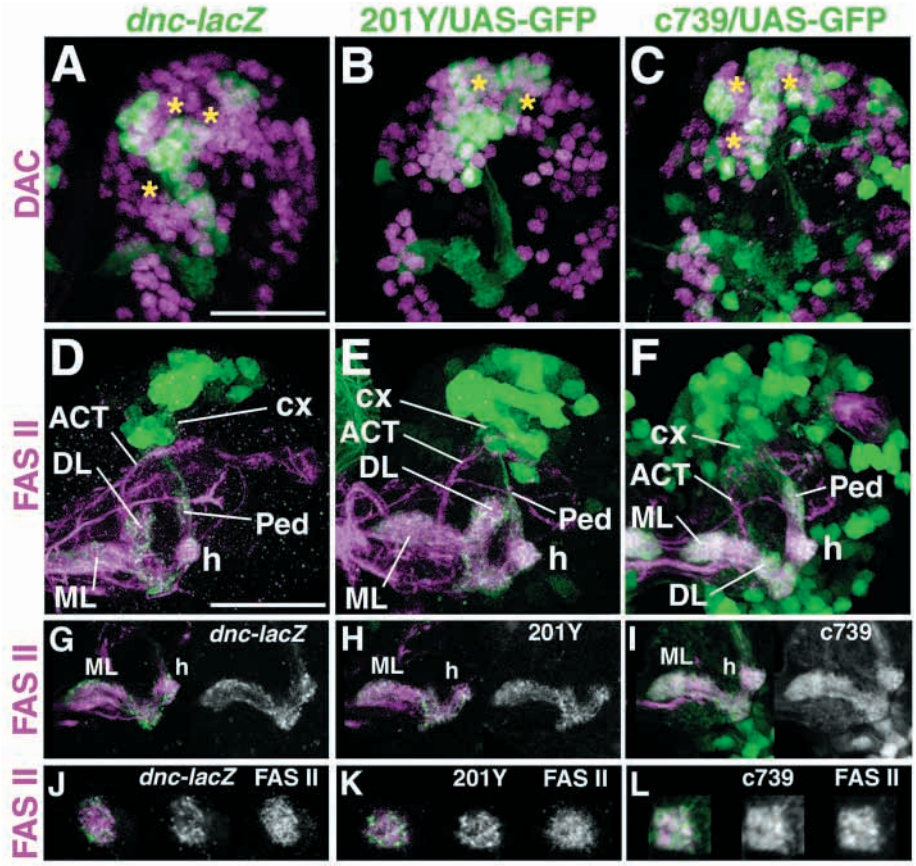


Fig. 3. Differential expression of MB markers in the first instar brain. (A,D,G,J) Expression of *dnc-lacZ* is revealed by an anti-β-GAL antibody (green/white). (B,E,H,K) GFP expression driven by 201Y (green/white). (C,F,I,L) GFP expression driven by c739 (green/white). (A-C) Slightly oblique dorsal views of MBs showing the Kenyon cells labeled with an anti-DAC antibody (magenta/white). Stars indicate the positions of the MB neuroblasts. (D-F) Lateral views showing the peduncle and lobes. Major axonal tracts, lobes and peduncle are labeled with an anti-FAS II antibody (magenta/white). Note that FAS II is expressed in the lobes and the distal part of the peduncle. (G-I) Single optical sections of the medial lobes. Marker expression is shown on the right in single channel. (J-L) Single optical sections of the dorsal lobes. Marker and FAS II expressions are shown on the right in single channels. Similar internal staining was obtained for peduncle sections (not shown). Scale bars: in A, 20 μm for A-C; in D, 20 μm for D-F. ACT, antennocerebral tract; cx, calyx; DL, dorsal lobe; h, heel; ML, medial lobe; Ped, peduncle.

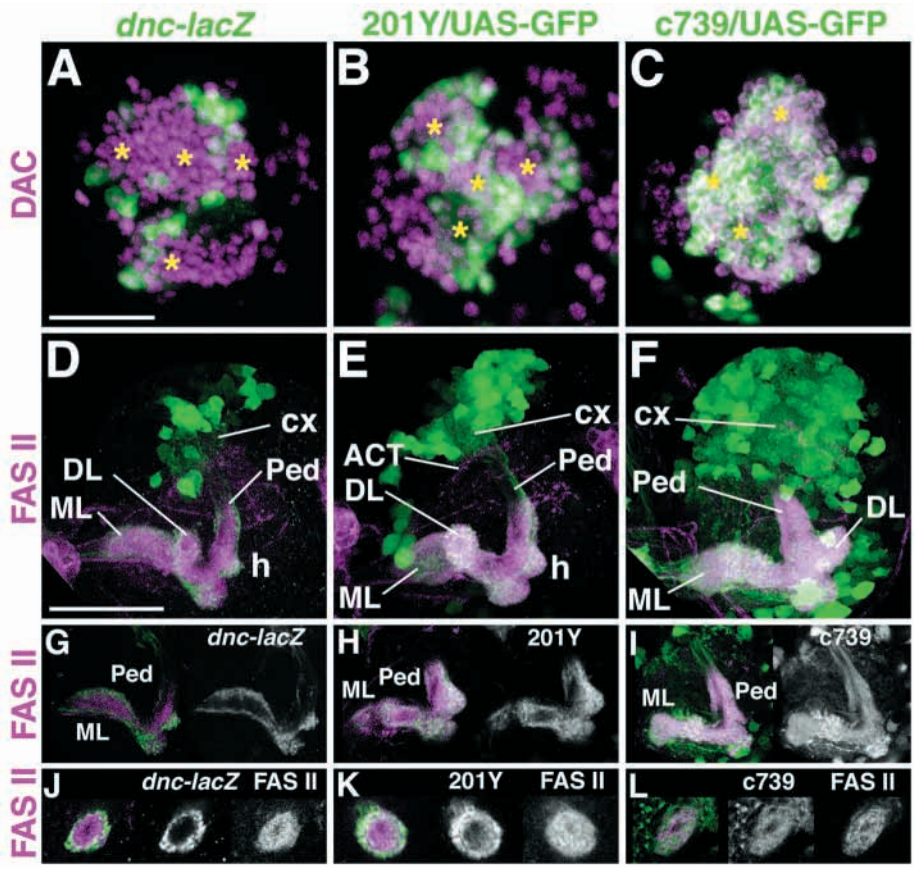


Fig. 4. Differential expression of MB markers in the second instar brain. (A,D,G,J) Expression of *dnc-lacZ* (green/white). (B,E,H,K) GFP expression driven by 201Y (green/white). (C,F,I,L) GFP expression driven by c739 (green/white). (A-C) Dorsal views of the Kenyon cell clusters labeled with an anti-DAC antibody (magenta/white). Stars indicate the positions of the MB neuroblasts. (D-F) Lateral views. The peduncles and lobes are labeled with an anti-FAS II antibody (magenta/white). (G-I) Single optical sections of the peduncles and the medial lobes. Marker expression is shown on the right in single channel. (J-L) Single optical sections of the dorsal lobes. Marker and FAS II expressions are shown on the right in single channels. Note similar internal staining for the peduncles and the lobes. Scale bars: in A, 25 μm for A-C; in D, 25 μm for D-F. ACT, antennocerebral tract; cx, calyx; DL, dorsal lobe; h, heel; ML, medial lobe; Ped, peduncle.

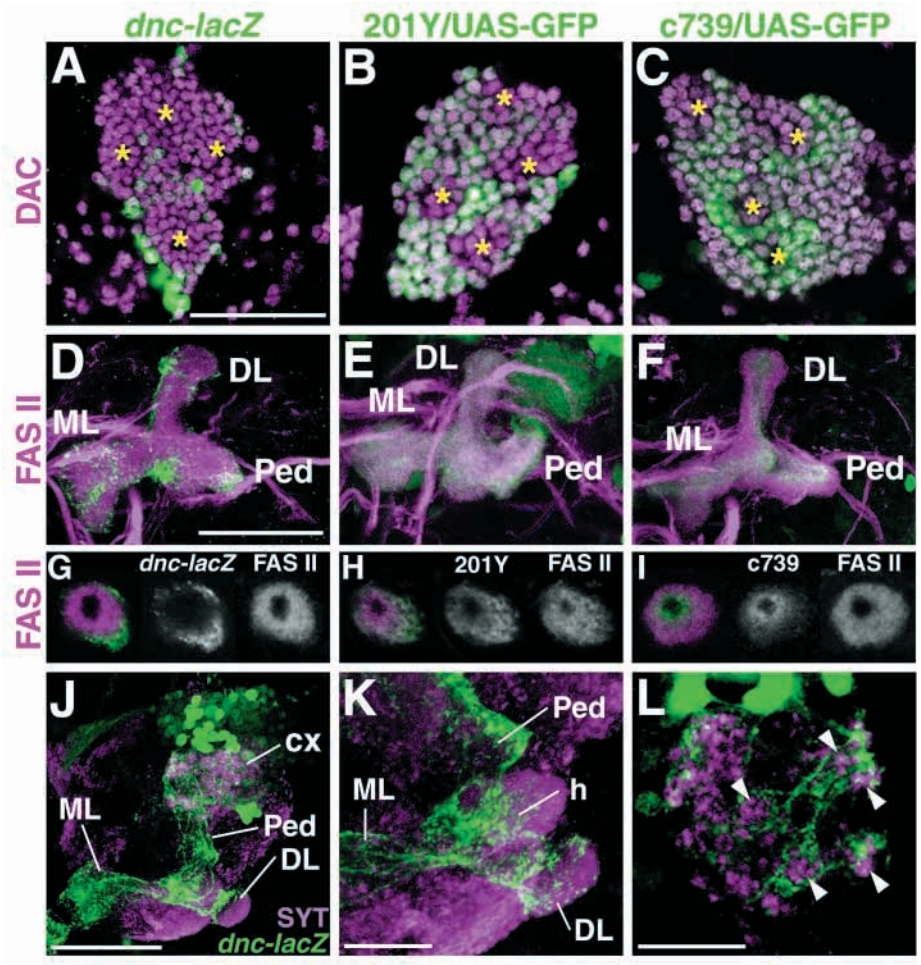


Fig. 5. Differential expression of MB markers in the third instar brain. (A,D,G) Expression of *dnc-lacZ* (green/white). (B,E,H) GFP expression driven by 201Y (green/white). (C,F,I) GFP expression driven by c739 (green/white). (A-C) Dorsal views of the Kenyon cell clusters of wandering third-instar larvae labeled with an anti-DAC antibody (magenta/white). Single optical sections. Stars indicate the positions of the MB neuroblasts. (D-F) Lateral views showing the lobes and the distal part of the peduncles. Reconstruction of optical sections. (G-I) Single optical sections of the peduncles. The lobes and the peduncle are labeled with an anti-FAS II antibody (magenta/white). Note similar internal staining for the peduncles and the lobes. Expression of 201Y and c739 was monitored with *UAS-GFP*. FAS II expression is weak in the innermost axons labeled with c739 and absent in the core. (J) Lateral view of a third instar MB double labeled with anti-Synaptotagmin (SYT; magenta/white) and anti-β-gal (green/white) antibodies. Reconstruction of optical sections. (K) High-magnification view of the dorsal lobe and heel showing a patchy distribution of the *dnc-lacZ* terminals in J. Single optical section. (L) A high power view of the dendritic arborization (J) of the *dnc-lacZ* neurons in the calyx. Note the extensive arborization of the *dnc-lacZ* neurons around the globular synaptic terminals (arrowheads). Single optical sections. Scale bars: in A, 25 μm for A-C; in D, 50 μm for D-F; 50 μm in J; 20 μm in K,L. ACT, antennocerebral tract; cx, calyx; DL, dorsal lobe; h, heel; ML, medial lobe; Ped, peduncle.

and lobes (Fig. 5F,I). By contrast, *dnc-lacZ* was maintained in the peripheral subdivisions both in the Kenyon cells (Fig. 5A) and their projections (Fig. 5D,G). Double staining with anti-FAS II antibody confirmed discrete internal organization of the peduncle and lobes, which are concentrically subdivided into at least three layers surrounding a core that was not labeled with the MB markers, including FAS II (summarized in Fig. 10C).

Interestingly, the reporter molecule for *dnc-lacZ* exhibited a characteristic patchy appearance in the calyx, peduncle and lobes, suggesting uneven distribution of the *dnc-lacZ* fibers (Fig. 5J,K). Indeed, higher magnification of the calyces double labeled with anti-β-gal and anti-synaptotagmin antibodies revealed extensive arborization of the *dnc-lacZ* expressing neurons around the synaptic terminals, which are likely to represent the afferent terminals of axonal collaterals of the antennocerebral neurons (arrowheads in Fig. 5L).

Characterization of the core fibers

While all the *GAL4* markers failed to drive expression in the core of the peduncle and lobes, we found that phalloidin, which binds filamentous actin, heavily stained the core (Fig. 6A). These core fibers are reminiscent of filopodia in their actin filament organization but extend long distance through the peduncle and split into the dorsal and medial lobes. Interestingly, at the calycal level, we found four bundles of the core fibers with surrounding axons that originated from the quadruple Kenyon cell clusters (Fig. 6B). These separate bundles converged into a single tract below the calyx forming the proximal part of the peduncle (Fig. 6C). The core fibers were not stained even with a pan-neural driver, *elev-GAL4* (Fig. 6E), unless a membrane-bound reporter (*mCD8::GFP*) was used (Fig. 6F), suggesting that the actin-rich fibers are tightly packed in the core. The high density of the actin-rich fibers might have enhanced the membrane-bound staining; alternatively, the core fibers are very thin with limited cytoplasmic space, and this might have hindered diffusion of the cytoplasmic reporter protein. The core fibers also were stained with OK107 (Connolly et al., 1996), which drives *GAL4* expression specifically in the Kenyon cells (Fig. 6G,H). These results suggest that the core is constituted of densely packed thin fibers that originated from a set of Kenyon cells. On the other hand, the core fibers were not stained with 201Y even with the membrane-bound GFP (Fig. 6I), confirming the expression pattern observed with the cytoplasmic GFP.

Sequential generation of the MB neurons and their projections

Having characterized the internal organization of the larval MBs, we then asked whether newly born Kenyon cells send out their axons into a particular layer or randomly into all layers. For this purpose, we used the MARCM mosaic

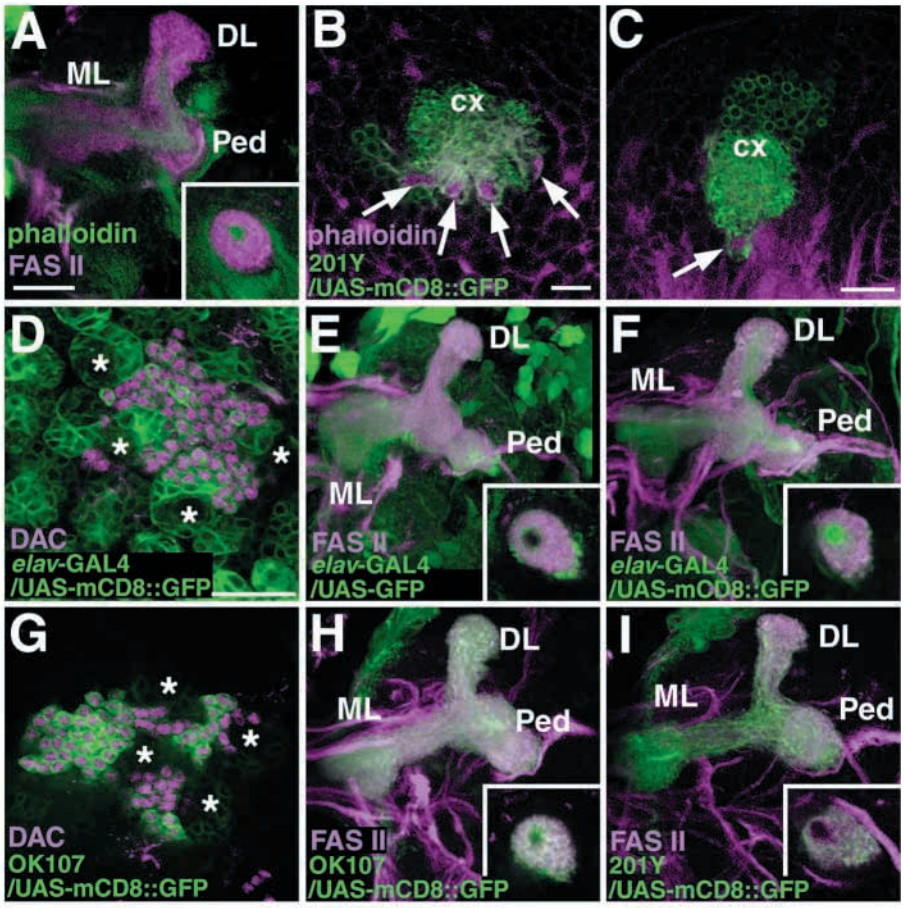


Fig. 6. Characterization of the core fibers. (A) Lateral view of wandering third instar MB labeled with an anti-FAS II antibody (magenta/white) and phalloidin (green/white). (B,C) MB axonal fibers around the calyces. MBs are labeled with *UAS-mCD8::GFP* (green/white) driven by 201Y and phalloidin (magenta/white). Single optical sections of wandering third instar MBs. (D-I) GFP reporter staining in wandering third instar MBs. *GAL4* drivers are (D-F) *elav-GAL4*, (G,H) OK107 and (I) 201Y. OK107 is expressed moderately in the MB neuroblasts and GMC, and strongly in the differentiated Kenyon cells. The reporters are (D,F-I) *UAS-mCD8::GFP* (green/white) and (E) *UAS-GFP* (green/white). (D,G) Dorsal views of Kenyon cells labeled with anti-DAC antibody (magenta); stars indicate the positions of the neuroblasts. (E,F,H,I) Lateral views of the lobes and the distal part of the peduncle labeled with anti-FAS II antibody. Note the core staining with the *elav-GAL4* and OK107 drivers. Insets are cross sections of peduncles. Scale bars: in A, 20 μm for A,E,F,H,I; 20 μm in B,C; in D, 20 μm for D,G. ACT, antennocerebral tract; cx, calyx; DL, dorsal lobe; h, heel; ML, medial lobe; Ped, peduncle.

technique (Lee and Luo, 1999), which allows temporally controlled generation of GFP labeled clones among the Kenyon cell population. When neuroblast clones were induced in the first instar stage and analyzed in the late third instar stage, they occupied proximal to distal positions to the original neuroblasts in 15 out of 15 cases (Fig. 7A) sending their axons into the core and surrounding layers (Fig. 7D). However, all two-cell clones (six out of six cases) induced in the first instar stage located distally to neuroblasts with axonal projections peripherally located in the peduncle and lobes (Fig. 7B,E). Lastly, all neuroblast clones (five out of five cases) induced at the beginning of the third instar stage formed a small cell

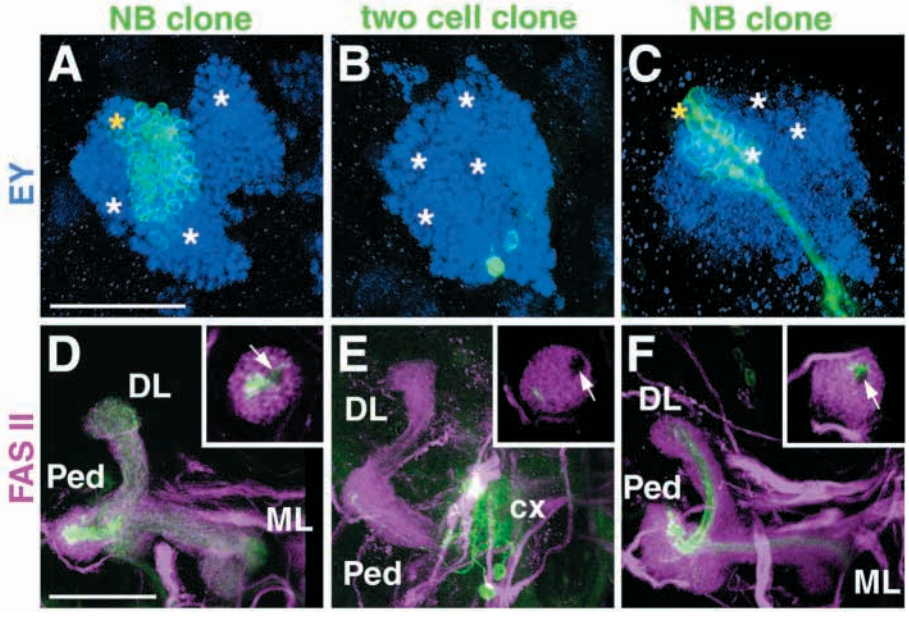


Fig. 7. Sequential generation of the MB neurons and their projections. (A,D) Neuroblast clone (green/white) induced in the first instar. (B,E) Two-cell clone induced in the first instar. (C,F) Neuroblasts clone induced at the beginning of the third instar. Clones are labeled with *elev-GAL4* and *UAS-mCD8::GFP*. (A-C) Dorsal views of the Kenyon cells labeled with anti-EY (blue). Late third instar stage. Stars indicate the location of the four-MB neuroblasts. The position of the GFP marked neuroblast is labeled with a yellow star. (D-F) Lateral views showing the peduncle, dorsal and medial lobes. Insets in D-F are optical cross sections of the peduncle. The peduncle and lobes are labeled with anti-FAS II (magenta). Arrow in the inset indicates the core. DL, dorsal lobe; cx, calyx; ML, medial lobe; Ped, peduncle. Scale bars: in A, 40 μm in A-C; in D, 40 μm for D-F. Genotypes: $GAL4^{c155}$, *hs-FLP/X* or *Y; G13, UAS-mCD8::GFP/G13, tubP-GAL80*.

Table 1. Larval MB defects in hypomorphic *fas II* mutants

Mutants	Categories of MB defects (%)			Number of MBs examined
	Dorsal lobe malformation	Medial lobe fusion	Calyx expansion	
fas II^{e76}				
Sample 1	19	0	0	36
Sample 2	28	20	0	40
Sample 3	2	17	0	58
Total	14	13	0	134
fas II^{e86}				
Sample 1	8	6	0	36
Sample 2	22	6	22	32
Sample 3	6	44	0	54
Total	11	23	6	122

cluster that was proximally positioned to the neuroblast (Fig. 7C). The axons of such newly formed cells projected into the core (Fig. 7F).

Structural MB defects in loss-of-function *fas II* mutants

Whereas FAS II is expressed in the vicinity of the growing MB axons in the embryonic brain, FAS II expression was not detected in the peduncles and lobes until the late embryonic stage 17 (data not shown) (Noveen et al., 2000). In order to examine the functional significance of FAS II in embryonic MB development, we examined a *fas II* null mutant, *fas II*^{eb112} (Grenningloh et al., 1991). Consistent with the late onset of FAS II in the embryonic MBs, the initial growth of the pioneer MB axons was not disturbed in *fas II*^{eb112} mutant (Fig. 8A). After converging at the protocerebral neuropil, the MB axons exhibited a normal medial turn and formed the primordial medial lobe (Fig. 8B).

As FAS II is expressed at high levels in the larval MBs, we then investigated larval MB development in viable hypomorphic *fas II* mutants (Grenningloh et al., 1991). Partial expression of the FAS II protein at 10% level in *fas II*^{e76} caused abnormal lobe morphology in 14% of cases (Fig. 8C). Similarly, partial expression of FAS II at 50% in *fas II*^{e86} resulted in gross structural defects of the lobes and calyxes (Fig. 8D). Notably, in both hypomorphic mutants, we observed variable degrees of phenotypic penetrance between independent samples, suggesting influences of other genetic and/or epigenetic factors (Table 1).

In order to examine whether FAS II is required intrinsically, we generated *fas II* null mutant clones by the MARCM technique (Lee and Luo, 1999) that allows visualization of the axonal projections of the mutant clones (Fig. 8E,F). No gross abnormality was found. However, contrary to the coherent axon bundle of the wild-type MB clones (see Fig. 7D inset), we found dispersed axonal fascicles in 23% (five of 22) of cases, in which axon bundles were randomly scattered throughout all axonal layers of the peduncle and lobes (Fig. 8F inset).

Disruption of the MB development by ectopic expression of FAS II

As demonstrated above, FAS II expression is temporally and spatially controlled during the MB development. Having examined loss-of-function phenotypes, we determined whether ectopic overexpression of FAS II causes abnormalities in MB development by crossing *UAS-fas II* flies (Lin et al., 1994) with *GAL4* drivers of different temporal and spatial expression patterns. Ubiquitous overexpression of FAS II in mature

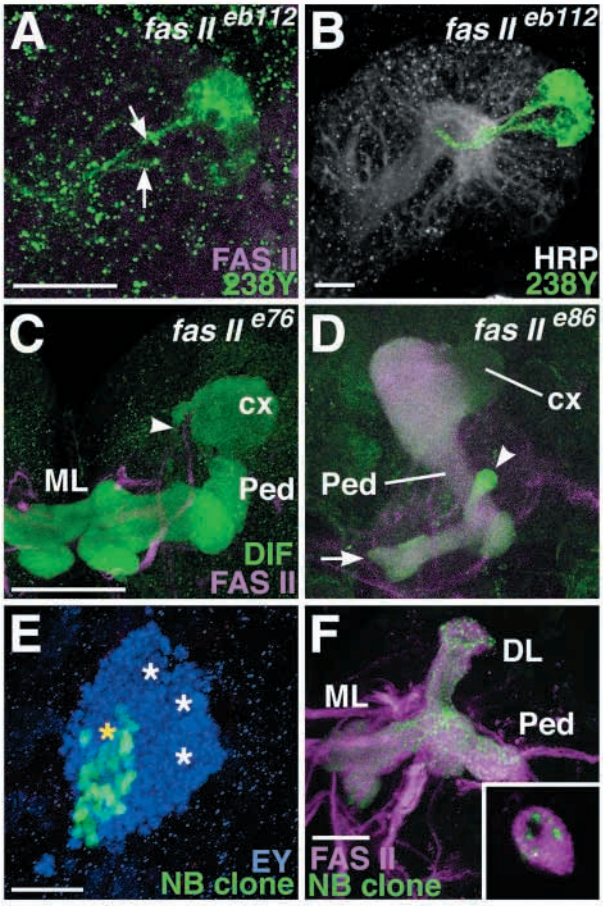


Fig. 8. Structural MB defects in loss-of-function *fas II* mutants. (A) Axonal projections of embryonic MB primordium in fas *II*^{eb112} mutant (protein null). Lateral view of the embryonic brain at late stage 16. FAS II (magenta) and 238Y (green). Single optical section. The embryonic MBs are labeled with *UAS-tau-lacZ* and 238Y-*GAL4*. Arrows indicate the growing MB axons. (B) Overview of an embryonic MB primordium in *fas II*^{eb112}. Reconstruction of optical sections. Same embryo as in A. Neurons are labeled with anti-HRP antibody (white). 238Y signal (green) is selectively enhanced. (C,D) Third instar larval MBs double labeled with anti-FAS II (magenta/white) and anti-DIF (green/white), which stains MB structures (Cantera et al., 1999). (C) *fas II*^{e76} hypomorphic (10%) mutant. Note the thin dorsal lobe (arrowhead) and fusion of the medial lobes. (D) *fas II*^{e86} hypomorphic (50%) mutant. Note the small dorsal lobe (arrowhead) and the medial lobes (arrow). Calyces are markedly expanded with aberrant accumulation of the FAS II protein. (E,F) *fas II*^{eB112} mutant clones at the late third instar stage (green/white). Mitotic recombination was induced in the first instar stage. (E) Dorsal view of the Kenyon cells labeled with anti-EY (blue). Stars indicate neuroblasts. (F) Lateral view showing the peduncle, dorsal and medial lobes labeled with anti-FAS II (magenta/white). Inset shows a cross section of the peduncle. The core fibers are not labeled owing to the driver/reporter combination used for the generation of the mosaic clones. Genotype used: *hs-FLP, tubP-GAL80, FRT19A/ fas II*^{eB112}*, FRT19A; 201Y/ UAS-GFP-T2*. cx, calyx; DL, dorsal lobe; ML, medial lobe; Ped, peduncle. Scale bars: 10 μm in A,B; in C, 50 μm for C,D; 20 μm in E,F.

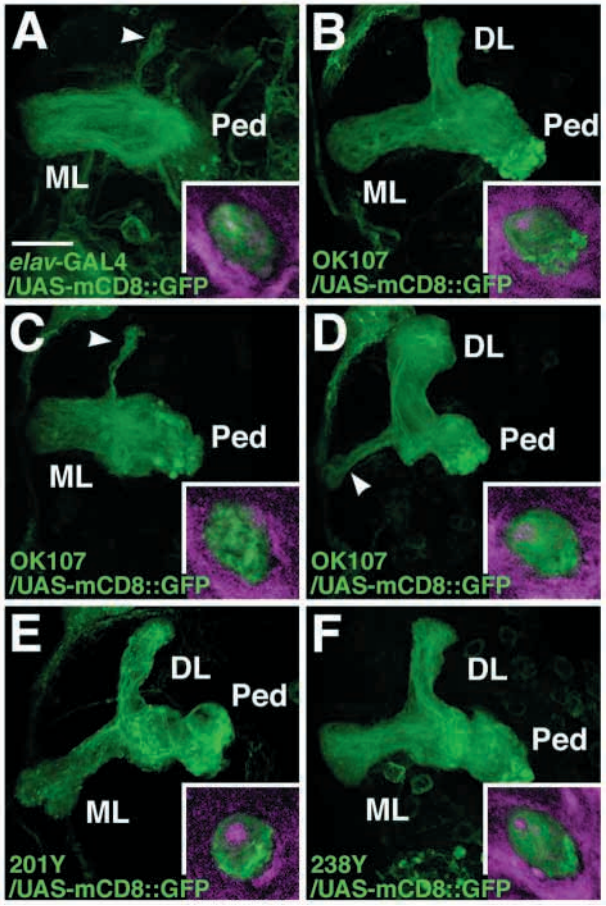


Fig. 9. Structural MB defects caused by overexpression of FAS II. Lateral views of larval MBs at wandering third instar stage. *GAL4* drivers are (A) *elav-GAL4*, (B-D) OK107, (E) 201Y and (F) 238Y. Reporters are *UAS-mCD8::GFP* in all cases (green). Insets show cross sections of peduncles with phalloidin staining (magenta). Arrowhead in A, malformed thin dorsal lobe. (B) MB with type 1 defects (Table 2). Note the blunt medial lobe as compared with the wild-type medial lobe in F, which terminates in three blobs. (C) MB with type 2 defects. Note the malformed thin dorsal lobe (arrowhead). (D) MB with type 3 defects. Note the malformed medial lobe (arrowhead) and expansion of the dorsal lobe. Scale bar: 20 μm. DL, dorsal lobe; ML, medial lobe; Ped, peduncle.

neurons with the *elev-GAL4* driver caused severe morphological abnormalities in the majority of the third instar MBs (Fig. 9A; Table 2). Both the dorsal and medial lobes were markedly affected. Furthermore, immunostaining with phalloidin revealed disruption of the core (Fig. 9A, inset). Localized overexpression of FAS II in MBs with OK107 also caused severe developmental defects in the majority of MBs examined (Fig. 9B-D; Table 2). However, we found no MB abnormality using 201Y despite its robust expression in the larval MB layers surrounding the core (Fig. 9E). As both *elev-GAL4* and OK107 drive FAS II expression from the middle embryonic stage before the onset of the endogenous FAS II, we asked whether similar defects could be caused by premature embryonic expression of FAS II using the MB-*GAL4* driver 238Y, which expression starts at mid-embryonic stage but otherwise is very similar to that of 201Y in the larval stages. Intriguingly, 238Y driven overexpression caused only mild

Table 2. Larval MB defects caused by *fas II* overexpression

	Categories of MB defects (%)			Total	
GAL4 driver	Type 1	Type 2	Type 3	Defective MBs (%)	Number of MBs examined
elav-GAL4	64	21	3	88	33
OK107	50	25	8	83	40
201Y	0	0	0	0	38
238Y*	13	4	0	17	24

Type 1, single blob medial lobe and almost normal dorsal lobe; type 2, single blob medial lobe and malformed thin dorsal lobe; and Type 3, malformed small medial lobe and expanded dorsal lobe.

*Mild defects. Medial lobes terminate in single-blob but internally harbor three branches.

lobe defects with low expressivity (Fig. 9F), suggesting that ectopic overexpression of FAS II in the core fibers of the larval MBs is the major cause for the severe disruption of the lobe system.

DISCUSSION

Studies of MB development with mosaic clones have shown that MB neurons in the adult brain are classified into three groups that project dorsally to the α and α′ lobes and medially to the β, β′ and γ lobes (Crittenden et al., 1998). Based on this classification, all the Kenyon cells born before the mid-third larval instar belong to the γ group (Lee et al., 1999). Only in the late third instar, the second group of neurons projecting into the α′ and β′ lobes is produced. In this study, using various MB markers, we have demonstrated that the larval Kenyon cells can be further subdivided into distoproximal concentric groups surrounding each of the neuroblasts. Furthermore, we have shown that the axonal projections of the Kenyon cells are also organized into concentric layers in the peduncle and lobes. Axons of newly born Kenyon cells project into the core that is constituted of densely packed thin fibers rich in actin filaments (Fig. 10B,C).

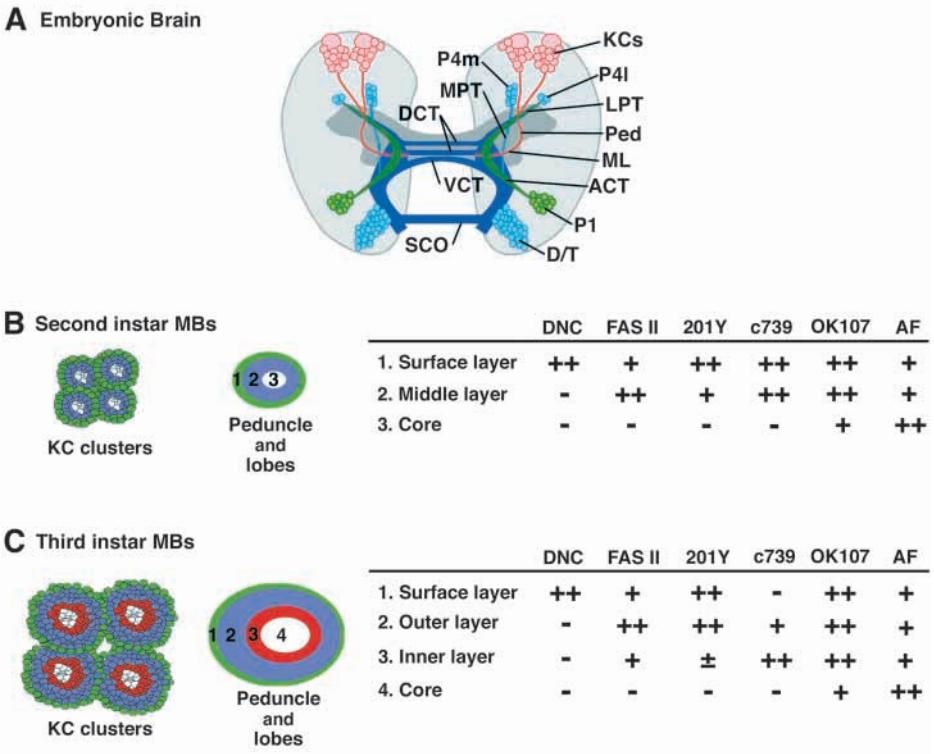
Generation of the larval MB layers

Previously, we have described distoproximal expression patterns of nuclear regulatory genes in the larval MB cell clusters (Kurusu et al., 2000). In particular, whereas *ey* is expressed in all the MB cells, including the neuroblasts and ganglion mother cells (GMCs), *dac* is expressed in differentiated Kenyon cells but not in the centrally located proliferating cells. *GAL4* MB markers, such as 201Y and c739, are expressed in a outer group of the differentiated Kenyon cells that are located several cell diameters away from the proliferating neuroblasts (Fig. 5) (Kurusu et al., 2000).

Based on these expression profiles of nuclear regulatory genes and *GAL4* markers in the cell bodies, we suggest that the Kenyon cells that are labeled with both DAC and 201Y project their axons into the concentric layers that also are labeled with FAS II. However, the proximally located Kenyon cells that are labeled with DAC but not 201Y may correspond to the newly differentiated MB neurons that project thin fibers into the core of the peduncle and lobes. Recently, by using a DsRed variant, Verkhusha et al. (Verkhusha et al., 2001) described a similar

concentric generation of Kenyon cell fibers in the surrounding layers of the peduncle and lobes, in which younger axons extend into the inner layer to shift older fibers into the outer layers. Our clonal studies on the larval projection patterns support this temporal order of layer generation and further show that axons of the newly produced Kenyon cells firstly project into the core as actin-rich thin fibers to shift to the surrounding layers as they undergo further differentiation.

Using electron microscopic studies, Technau and Heisenberg (Technau and Heisenberg, 1982) described a bundle of thin fibers centrally located in the peduncle and lobes, and postulated that the central fibers might be a set of larval fibers that remain throughout metamorphosis as a guide for the ingrowing imaginal fibers. Our results are consistent with this hypothesis and further demonstrate that the core fibers are derived from newly produced Kenyon cells to undergo a dynamic translocation to the surrounding layers as they differentiate. As the adult α′ and β′ neurons are generated in the late larval stage (Lee et al., 1999) and may project into the larval MB core, we presume that their axons might remain as premature core fibers during the early stages of metamorphosis.



	DNC	FAS II	201Y	c739	OK107	AF
1. Surface layer	++	+	++	++	++	+
2. Middle layer	-	++	+	++	++	+
3. Core	-	-	-	-	+	++

	DNC	FAS II	201Y	c739	OK107	AF
1. Surface layer	++	+	++	-	++	+
2. Outer layer	-	++	++	+	++	+
3. Inner layer	-	+	±	++	++	+
4. Core	-	-	-	-	+	++

Fig. 10. (A) The MB primordia in the embryonic brain. Frontal view of the embryonic brain at late stage 16. Only major tracts are shown. Optic lobes are not included. (B,C) Layer organization of the second and third instar larval MBs. Dorsal images of Kenyon cell clusters and cross sections of the peduncle and lobes. Corresponding subdivisions are shown in same colors. Relative expression levels of various MB markers are summarized in the table. The second instar MBs can be concentrically subdivided in two layers surrounding the core. With increase in the numbers of the Kenyon cells and their projections, the third instar MBs can be subdivided into three layers surrounding the core. Note that the distoproximal concentric subdivisions of each of the four Kenyon cell clusters topologically correspond to the unified concentric subdivisions in the lobes and the peduncle. The core consists of a bundle of newly formed axon fibers that contain densely packed actin filaments. AF, actin filaments; ACT, antennocerebral tract; DCT, dorsal commissural tract; DNC, *dnc-lacZ*; KCs, Kenyon cells; LPT, lateral protocerebral tract; MPT, medial protocerebral tract; P1, P4l, P4m, and D/T, fiber tract founder clusters (Nassif et al., 1998); Ped, peduncle; VCT, ventral commissural tract.

Layer subdivisions of MBs have been described in other insects. The MB lobes of the honey bee *Apis mellifera* are subdivided into discrete layers (Schäfer et al., 1988; Schürmann and Erber, 1990; Bicker, 1991), which correspond to the stratified arrangements of dendritic trees of efferent neurons (Rybak and Menzel, 1993; Rybak and Menzel, 1998; Strausfeld, 1999). In the cockroach *Periplaneta americana*, afferent terminals segment the calyces into four discrete zones, which receive afferents from distinct sets of olfactory glomeruli (Strausfeld and Li, 1999). In the *Drosophila* larval MBs, it is yet to be seen how the layers of the peduncle and lobes are represented in the calyces and whether the layer subdivisions correspond to functional compartments that are wired to different sets of afferent interneurons of the larval antennal lobes. The dendritic arborization in the *Drosophila* larval calyces is highly condensed and hinders direct anatomical examination of its internal structure. We speculate that micro subdivisions that are beyond the anatomical resolution achieved in this work could exist in the calyces as well.

Functions of FAS II in MB development

The FAS II protein, a member of the Ig superfamily, mediates axon fasciculation through homophilic adhesion (Grenningloh et al., 1991). In the *Drosophila* ventral nerve cord, FAS II is expressed on a subset of embryonic axons, many of which selectively fasciculate in three distinct longitudinal axon pathways (Grenningloh et al., 1991; Lin et al., 1994). In *fas II* loss-of-function mutants, the axons that normally fasciculate together in the three FAS II pathways fail to do so and these axon fascicles do not form (Lin et al., 1994). However, overexpression of the FAS II protein results in a gain-of-function phenotype in which pairs of pathways that should normally remain separate instead become abnormally joined together, indicating that FAS II controls specific patterns of selective fasciculation and axon sorting in the central nervous system. Notably, FAS II is not required for several aspects of growth cone guidance: despite the severe defects in fasciculation, the follower growth cones find their way normally in the ventral nervous system (Lin et al., 1994).

These in vivo functions of FAS II in the ventral nerve cord correspond with the loss-of- and gain-of-function phenotypes in the developing MBs. Although the axons of the embryonic

MBs initially grow along the FAS II-expressing cells, they can find their pathways in the developing brain in the absence of the FAS II protein. Later in the larval stages, decreases in FAS II protein level result in abnormal development of the lobes and the internal layers. Particularly noteworthy is that FAS II is intrinsically required for the clonal integrity of the axonal fascicles and hence generation of correct organization of the internal layers. Contrary to our findings, using a similar but independent set of hypomorphic *fas II* alleles, Cheng et al. (Cheng et al., 2001) have described lack of morphological defects in the adult MBs at the levels of conventional and electron microscopy. Although the existence of internal defects in the adult MBs cannot be ruled out, the discrepancies could also be accounted for by alternative genetic backgrounds, which is particularly influential to the expressivity of anatomical defects in the adult brain (de Bell and Heisenberg, 1996).

In the course of MB development, FAS II becomes detectable at late stage 17 in the embryonic peduncle and lobes (Noveen et al., 2000) (M. K. and K. F. T., unpublished). Later in the larval stages, FAS II is expressed in the MB layers but not in the core fibers. The robust gain-of-function phenotypes caused by the ectopic overexpression of FAS II argue for the functional importance of this temporal and spatial regulation of FAS II expression in MB development. Ectopic FAS II expression with OK107 in MB axons including the newly generated core fibers results in major disruption of the branching patterns of the lobes whereas overexpression with 201Y only in the surrounding layers results in no abnormality. These results on the FAS II functions emphasize the importance of cell adhesion properties for the correct branching of the MB lobes and the development of the internal axonal layers.

Beyond *Drosophila* MBs

It has long been appreciated that the mammalian cerebral cortex is organized into layers, which are connected to different functional neural circuits (Chenn et al., 1997). Neurons in different layers are generated at different stages during development and migrate away from the ventricular zone, where they are generated. As a result, the deepest layers are formed by neurons born at early stages and the more superficial layers are formed by neurons that are born later and migrate past the deep layers. Similarly, during vertebrate retinal development, six types of neurons and one type of glia are orderly generated and form discrete layers (Livesey and Cepko, 2001).

In this study, we have shown that the *Drosophila* MB cell bodies and their axonal projections are organized into layers. Furthermore, there is a temporal sequence in layer formation, in which younger neurons project first into the core to shift to the surrounding layers as they differentiate. Unlike the mammalian cortex, neuronal migration has not been demonstrated so far in MB development. However, MB neurons originating from the quadruple Kenyon cell clusters initially form four axon bundles, yet eventually converge into a single tract in the peduncle: a process that calls for dynamic sorting and fasciculation of the growing axons during the formation of the ordered internal layers of the peduncle and lobes. The identification of cell adhesion molecules that underlie this process is a subject for intriguing future investigation. Determining how different type neurons are orderly generated and allocated to different topographical subdivisions is enormously important for developmental neuroscience. We anticipate that studies of the MB development in the *Drosophila* brain will lead to important insights into the molecular mechanisms that control the sequential generation of neurons and their positioning into layers during the development of both vertebrate and invertebrate brains.

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