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, I 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 MB neuroblasts and their progenies, which locate at the anterior region of the brain in neuraxis (Fig. 3A; Tettamanti *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. 3B) 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. 3C) 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. 3D, 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. 3E; see also the

schematic presentation of embryonic tracts in Fig. 4). By late stage 17, the dorsal lobe emerged as a collateral outgrowth at around the turn; the MB axons start to express FAS II; calyces emerged only near the end of embryogenesis (Tettamanti *et al.*, 1997; Noveen *et al.*, 2000).

Expression of nuclear regulatory genes in the embryonic MB primordia

Based on the neuroanatomical studies of embrynionic MB primordia, I searched for putative regulatory factors, and found that EY is expressed in the embryonic MB primordia (Fig. 5A). High-resolution image shows that EY is expressed in the MB neuroblasts, ganglion mother cells and their progenies suggesting pivotal functions of EY in various stages of cell differentiation in MB development (Fig. 5B and Fig. 7). Intriguingly, analyses of embryonic brains double labeled with anti-EY and anti-FAS II antibodies revealed that EY is expressed in not only the MB but also the deutocerebrum anlagen, the latter of which gives rise to the antennal lobe (arrow in Fig. 5C). A prominent tract connecting the MB primordium and the deutocerebral cell cluster, ACT, was already established by late embryonic stage 14 connecting the MB primordium and the protocerebral cell cluster.

In addition to *ey*, studies on *Drosophila* eye development have revealed a cascade of regulatory genes that function synergistically in the early specification of eye primordia (Fig. 14A; Pignori *et al.*, 1997; Chen *et al.*, 1997; Halder *et al.*, 1998; Czerny *et al.*, 1999). Among such regulatory genes involved in eye development, I found that *toy* also is expressed in the embryonic MBs (Fig. 6A). Moreover, *dac*; another gene involved in eye development, also is expressed in the embryonic MBs (Fig. 6B; see also Fig 12A for higher magnification). However, the expression of the DAC protein is rather confined to ganglion mother cells and embryonic Kenyon cells (Fig. 12A). On the other hand, in

contrast to the eye development cascade, neither *sine oculis* (*so*; Cheyette *et al.*, 1994, Serikaku and O'Tousa, 1994) nor *eyes absent* (*eya*; Bonini *et al.*, 1993; 1997) is expressed in the embryonic MBs though EYA was detected in nearby cell clusters in the anterior region of the embryonic brain (Fig. 6C, D).

Expression of nuclear regulatory genes in the larval MBs

The characteristic expression of *ey, toy* and *dac* in the developing MBs are maintained in the larval brain. EY is expressed in all of the larval MB cells at significant level while the reporter gene expression of a *GAL4* line 201Y (Yang *et al.*, 1995), is absent in the central cells (Fig. 8A-C). Expression of *toy* is also evident in the Kenyon cells (Fig. 8D). As with *ey, toy* is expressed in all of the MB cells (Fig. 9). On the other hand, DAC is not expressed in the central cells including neuroblasts and ganglion mother cells while it is clearly detected in distantly located cells (Fig. 8E, F). Neither *so* nor *eya* is expressed in the larval MBs though they are expressed in nearby cells (Fig. 8G, H).

The differential expression of *ey, toy* and *dac* initially observed in the embryonic MBs are thus pronounced in the larval brain as the neuropil structures of the MBs are developed. The expression patterns of these genes in the Kenyon cells also are maintained in the pupal stage (Fig. 12G).

Structural defects in *ey* and *dac* mutants

The distinctive expression profiles of *ey, toy* and *dac* in the embryonic and larval MBs suggest combinatorial regulatory mechanisms in the initial formation and structural development of the MBs. In order to examine functional significance of these genes in the MBs, I examined the neural structures of the developing MBs in mutant backgrounds of either *ey* or *dac*.

As described in Fig. 1, the larval MBs are topologically similar to the adult MBs but have only two orthogonal lobes, dorsal and medial lobes (Tettamanti *et al.*, 1997; Crittenden *et al.*, 1998; Armstrong *et al.*, 1998). Internally, the peduncles and lobes have simple concentric organization, in which the FAS II (Crittenden *et al.*, 1998) and DIF (Cantera *et al.*, 1999) proteins are expressed homogeneously except for the central unstained core (Fig. 10A). Mutational inactivation of *ey* resulted in moderate defects in the larval MBs in all the cases examined with weak but consistent suppression of FAS II in the peduncles and lobes (Fig. 10B). The distribution of FAS II also is affected: the globular end of the dorsal lobe is often devoid of FAS II. On the other hand, a null mutation of *dac* (dac^4) barely affected the larval MBs (Fig. 10C). However, 50 % reduction of *dac* activity in heterozygous larvae enhanced the structural defects of *ey* mutants, suggesting synergistic regulatory functions of the two genes in the development of the MB structures (Fig. 10D). In the double mutant for *ey* and *dac*, most parts of the peduncles and lobes showed gross structural defects including significant malformation of the dorsal lobe in many cases (10-20%). Furthermore, FAS II expression is markedly suppressed leaving uneven residual expression in the peduncles and remaining lobes.

The significance of *ey* and *dac* in MB development was further examined in the early pupal stage, in which MBs undergo massive degeneration and reorganization to form the complex adult MB structures (Crittenden *et al.*, 1998; Armstrong *et al.*, 1998; Lee *et al.*, 1999). In 50 hours after puparium formation, most of the MB structures are reorganized into the adult architecture, in which FAS II is strongly expressed in the α/β lobes and peduncles, and moderately in the γ lobe (Fig. 11A). In addition, it is heavily expressed in the ellipsoid body, which belongs to the central complex. On the other hand, DIF is strongly expressed in the γ lobe and weakly in the other lobes and the peduncle. Mutations of *ey* abolished all the neuropil structures of the pupal MBs in all

the cases examined whereas Kenyon cells expressing DAC are retained (Fig. 11B). Notably, ellipsoid body also is disrupted in the mutant. The *dac* [4] mutation disrupted most of the neuropil structures of the pupal MBs (Fig. 11C), leaving Kenyon cells expressing EY protein intact. Occasionally *dac* [4] caused ectopic projections of peduncles (arrowheads in Fig. 11D). In these cases, the structural profile of the FAS II expression resembled that of the larval MB structures, with homogeneous concentric patterns suggesting failure of reorganization of the MB structures at the onset of pupation. Thus, these results clearly demonstrate the functional importance of *ey* and *dac* in the structural formation of the adult MBs in the course of the massive neural reorganization in the early pupal stage.

Independent regulation of *ey* and *dac* in MB development

Studies of eye development have revealed a combinatorial network of key regulatory genes, in which *toy* acts upstream of *ey*, which initiates the regulatory feedback loop that additionally includes *so, eya*, and *dac* (Fig. 14A; Czerny *et al.*, 1999). These nuclear regulatory genes then synergistically control the subsequent stages of eye development. To dissect the regulatory network of MB development, I examined expression of *ey, toy* and *dac* in various mutant backgrounds.

Whereas EY and DAC are clearly coexpressed in the embryonic primordia, *ey* expression was not affected by the loss of *dac* activity and vice versa (Fig. 12A-C). Likewise, expression of EY and DAC was independent of each other's activity in the larval MBs (Fig. 12D-F). EY and DAC are coexpressed in most of the Kenyon cells at the pupal stage except for the central cells which express only EY (Fig. 12G). Again, mutation of *ey* did not alter the DAC expression though the number of the Kenyon cells was slightly reduced (Fig. 12H). Mutation of *dac* did not alter EY expression at all with

normal number of Kenyon cells (Fig. 12I).

Expression of *toy* is initiated from the cellular blastoderm stage earlier than the onset of *ey* and *dac* in both the eye and brain (Czerny *et al.*, 1999). I examined DAC expression in *nullo 4* embryos, which lack both *ey* and *toy* genes due to loss of the fourth chromosome. Despite that the brain was largely deformed in *nullo 4* embryos, characteristic MB neuroblasts expressing a nuclear marker, *tailless* were found at a dorsolateral position of each brain hemisphere with DAC expressing progenies (Fig. 13). Taken together, in contrast to the intricate feedback cascade in eye development, these results argue for distinct parallel cascades for the regulation of *ey* and *dac* in the developing MBs (Fig. 14B).

Layer development in the larval MBs

While the four MB neuroblasts continue 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 follow the medial and the dorsal lobe projections that are pioneered at the embryonic stage with a concomitant increase in the sizes of the lobes. By contrast, I found that a set of genes are turned on in the Kenyon cells following hatching of the first instar larvae in slightly different patterns in both the cell bodies and their projections (Fig. 15). As development proceeded, these differential gene expression patterns became more evident in the second instar larval stage (Fig. 16). While the DAC protein is expressed in most of the Kenyon cells, *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. 16A and Fig. 20A for schematic presentation). Expression of 201Y was detected in another subset of cells located more centrally in each of the Kenyon cell clusters (Fig. 16B), whereas c739 showed a widespread expression in most

of the cells (Fig. 16C).

Remarkably, these differential expression patterns observed in the Kenyon cells were topologically reflected in their axonal projections in the peduncle and lobes (Fig. 16D-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; c739 was detected in most axons, a pattern similar to that of FAS II.

As development further proceed, further subdivisions emerged in the third instar larval stage with increasing number of Kenyon cells and their axons (Fig. 17). Moreover the expression patterns of 201Y and c739 markers changed in both cell bodies and their projections; 201Y was then detected in many on of the Kenyon cells (Fig. 17B) and their projections (Fig. 17E, H) obscuring its peripheral pattern in the previous larval instar; c739 was then detected in a group of cells located near each of the neuroblast (Fig. 17C). The axons of the c739 expression cells projected into an inner layers of the peduncle and lobes (Fig. 17F, I). In contrast, *dnc-lacZ* was maintained in the peripheral subdivisions both in the Kenyon cells(Fig. 17A) and their projections (Fig. 17D, 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. 20B).

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. 18A, B). 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. 18C).

Characterization of the core fibers

While all the *GAL4* markers failed to drive expression in the core of the peduncle and lobes, I found that phalloidin, which binds filamentous actin, heavily stained the core (Fig. 19A). 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, I found four bundles of the core fibers with surrounding axons that originated from the quadruple Kenyon cell clusters (Fig. 19B). These separate bundles converged into a single tract below the calyx forming the proximal part of the peduncle (Fig. 19C). The core fibers were not stained even with a pan-neural driver, *elev-GAL4* (Fig. 19E), unless a membrane-bound reporter (*mCD8::GFP*) was used (Fig. 19F), suggesting that the actin-rich fibers are tightly packed in the core. The high density of actin-rich fibers might have enhanced the membrane-bound staining. It might also be that the core fibers are very thin with limited cytoplasmic space that 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. 19G, H). These results suggest that the core is consisted 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. 19I), confirming the expression pattern observed with the cytoplasmic GFP.

Sequential generation of the MB neurons and their projections

Having characterised the internal organization of the larval MBs, I then asked whether

newly born Kenyon cells send out their axons into a particular layer or randomly into all layers. For this purpose, I utilised the MARCM mosaic 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 analysed in the late third instar stage, they occupied proximal to distal positions to the original neuroblasts in 15 out of 15 cases (Fig. 21A) sending their axons into the core and surrounding layers (Fig. 21D). On the other hand, all two-cell clones (6 out of 6 cases) induced in the first instar stage located distally to neuroblasts with axonal projections peripherally located in the peduncle and lobes (Fig. 21B, E). Lastly, all neuroblast clones (5 out of 5 cases) induced at the beginning of the third instar stage formed small cell clusters that was proximally positioned to the neuroblast (Fig. 21C). The axons of such newly formed cells projected into the core (Fig. 21F).

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 (Fig. 3), FAS II expression was not detected in the peduncles and lobes until the late embryonic stage 17 (Noveen *et al.*, 2000). In order to examine the functional significance of FAS II in the embryonic MB development, I 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. 22A). After converging at the protocerebral neuropil, the MB axons exhibited a normal medial turn and formed the primordial medial lobe (Fig. 22B).

Since FAS II is expressed at high level in the larval MBs, I 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 total 14% cases (Fig. 22C). Similarly, partial expression of FAS II at 50% in *fas II*^{e86} resulted in gross structural defects of the lobes and calyxes (Fig. 22D). Notably, in the both hypomorphic mutants, I 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, I 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. No gross abnormality was found (Fig. 22E, F). However, contrary to the coherent cluster of axon bundles of the wild type MB clones (Fig. 21D inset), I 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. 22F 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, I 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 neurons with the *elev-GAL4* driver caused severe morphological abnormalities in the majority of the third instar MBs (Fig. 23A and Table 2). Both the dorsal and medial lobes were markedly affected. Furthermore, immunostaining with phalloidin revealed disruption of the core (Fig. 23A inset). Localised overexpression of FAS II in MBs with OK107 also caused severe developmental defects in the majority of MBs examined (Fig. 23B-D and Table 2). On the other hand, I found no MB abnormality using 201Y despite its robust expression

in the larval MB layers surround the core (Fig. 23E). Since both *elev-GAL4* and OK107 drive FAS II expression from the middle embryonic stage before the onset of the endogenous FAS II, I 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 lobe defects with low expressivities (Fig. 23F), 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 systems.