DISCUSSION

Comparison of genetic networks in MB and eye development

In the development of eye (summarized in Fig. 14A), *ey* plays important functions as a master control gene. Targeted expression of *ey* is sufficient to induce the development of ectopic eyes on wings, legs and antennae (Halder *et al.*, 1995). Recent studies have revealed that *toy* regulate expression of *ey* through binding its eye specific enhancer sequences (Czerny *et al.*, 1999). *toy* is also able to induce ectopic eyes on various epidermal tissues. Both *toy* and *ey* are expressed in subsequent larval stages in the eye primordia. In addition to *ey* and *toy*, several genes encoding nuclear transcription factors are required for eye development and have been implicated to function synergistically. Targeted expression of *dac* or *eya* on their own induces ectopic eyes though albeit much less efficient than *ey* (Shen *et al.*, 1997; Bonini *et al.*, 1997). Combined expression of *so* and *eya* (Pignoni *et al.*, 1997) or *dac* and *eya* (Chen *et al.*, 1997) synergistically induces ectopic eyes with positive feed back stimulation on *ey*.

In the present study, I have shown that three of the eye development genes, *ey*, *toy*, and *dac*, are expressed in the developing MBs, and that *ey* and *dac* have pivotal functions in the structural formation of the MBs (Fig. 14B). However, in contrast to the regulatory cascade in eye development, two of the key regulators, *so* and *eya*, are not expressed in the developing MBs. Furthermore, I have shown that *ey* and *dac* are independently regulated in the MBs suggesting a distinct combinatorial code of regulatory genes and parallel cascades in the development of the MBs. While describing the expression of *toy* in the MBs, it was unsuccessful to reveal the relationship between *toy* and *ey*. It will be interesting to determine the function of *toy* in the genetic cascade controlling MB development.

Generation of the larval MB layers

Expression patterns of the nuclear regulatory genes are organized disto-proximally in the late-third instar larval MB cells (Fig. 9). In particular, *ey* is expressed in all the MB cells including the neuroblasts, GMCs and the Kenyon cells, whereas *dac* is expressed in differentiated Kenyon cells but not in the central proliferating cells. Moreover, double staining for DAC and the *GAL4* MB markers including 201Y and c739, demonstrated that these *GAL4* MB markers are expressed only in the outer group of the Kenyon cells that are located several cell diameters away from the neuroblasts (Fig. 20).

Based on these expression profiles of nuclear regulatory genes and *GAL4* markers in the cell bodies, I 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. On the other hand, 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. 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.

By electron microscopic studies, 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. My 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. Since 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, I presume that their axons might remain as

premature core fibers during the early stages of metamorphosis.

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). On the other hand, 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 mutant 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 internal layers, which may require correct axonal sorting and fasciculation of the growing axons of the distinct subsets of Kenyon cells. Particularly noteworthy is that FAS II is intrinsically required for the clonal integrity of the axon projections and hence generation of correct organization of the internal fascicles. Contrary to our findings,

using similar but independent set of hypomorphic *fas II* alleles, Cheng *et al.* (2001) 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). 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 with *elav-GAL4* and OK107 drivers argue for the functional importance of this temporal and spatial regulation of the FAS II expression in MB development. On the other hand, overexpression of FAS II with 201Y causes no abnormality in the larval MBs. Notably, in contrast to OK107, 201Y drives *GAL4* dependent gene expression in the larval layers excluding the core fibers (Fig. 19I). Although premature expression in the embryonic MBs with 238Y causes mild abnormalities, these results are consistent with the notion that ectopic FAS II expression in newly generated core fibers causes major disruption of the branching pattern of the lobe systems. These results on the FAS II functions emphasize the importnace of cell adhesion properties for the correct branching of the MB lobes and the development of the internal layers. Further elucidation of the processes that organize the highly ordered structures of the MBs may shed light on the mechanisms by which the precisely wired neural circuits are constructed during brain development.

Internal layer organization of MBs across taxa

Immunocytological studies of honey bee *Apis mellifera* brains have shown that, similar

to the organization of the *Drosophila* MBs, the *Apis* lobes are subdivided into discrete longitudinal layers (Schäfer *et al.*, 1988; Schürmann and Eber, 1990; Bicker, 1991), which correspond to the stratified arrangements of dendritic trees of efferent neurons (Rybak and Menzel, 1993, 1998; Strausfeld, 1999). In the cockroach *Periplaneta americana*, afferent terminals segment the calyces into four discrete zones, I, II, III and IIIA, which receive afferents from 1) two discrete populations of sexually isomorphic olfactory glomeruli, 2) two types of male-specific olfactory glomeruli, 3) the optic lobes, and 4) multimodal interneurons that originate in protocerebral neuropils, respectively (Strausfeld and Li, 1999a). The cockroach calyx neuropil arranged disto-proximally into four zones is transformed into laminar arrangements of their projections in the lobes (Strausfeld and Li, 1999b). Particularly noteworthy is that sexually isomorphic type2 projection neurons of the antennal lobes end restrictively in the calyx's zone I, suggesting that relative positions amongst the antennal glomeruli may be precisely mapped into different layer subdivisions of the MB calyces.

In accordance with functional subdivisions of the MBs similar to the cockroach MBs, the present study shows the layer subdivisions of the peduncles and lobes of the *Drosophila* MBs. In paticular, *dnc-lacZ* neurons exclusively belong to the outermost zone in the Kenyon cell clusters and extend their axons into the surface layer of the peduncle and lobes throughout the larval stages. At the calycal level, while describing the arborization of *dnc-lacZ* neurons in the calyces in a way that wraps and networks the synaptic terminals of the antennocerebral tract, I failed to demonstrate discrete internal subdivisions of the larval calyces. In the light of the relatively simple olfactory centers in the *Drosophila* larval brain (Stocker *et al.*, 1997), it is conceivable that internal subdivisions are yet to be formed in the larval calyces. However, it is also possible that the larval calyces are already organized into discrete subdivisions but at a highly

compact manner that might be beyond the anatomical resolution used in this work.

It has been proposed that diverged MBs across taxa can be derived from developmental stages of a basal archetype insect such as the cockroach *Periplaneta americana* (Strausfeld *et al.*, 1998). In *Periplaneta*, a laminar subdivision is added to the peduncle and lobes at each instar. In contrast, our results reveal that the underlying subdivisions of the *Drosophila* larval MBs are concentric and disto-proximally arranged. The adult MBs are longitudinally subdivided and restrictively invaded by the dendrites of efferent neurons (Yang *et al.*, 1995; Crittenden *et al.*, 1998). Strausfeld *et al.* (1999 and FlyBrain; http://flybrain.neurobio.arizona.edu/) posited that different types of MB subdivisions might have evolved by arrest of the developmental programs at different stages of the phylogenetic basal archetype. Indeed, the concentric and longitudinal subdivisions of the larval and the adult *Drosophila* MBs exhibit a close correspondence with the internal organizations of the first and second instar stages of the *Periplaneta* MBs.

Beyond the mushroom bodies

It has long been appreciated that the mammalian cerebral cortex is organized into layers, which are connected to different functional neural circuits (reviewed in 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 (reviewed in Livesey and Cepko, 2001).

In this study, I 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. On the other hand, 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 of intriguing future investigation.

It has been suggested that olfactory pathways of arthropod and vertebrate are anatomically related (Fig. 24; Strausfeld *et al.*, 1998; Strausfeld and Hildebrand, 1999). MBs receive primary inputs from the antennal lobes, which are the equivalent of the vertebrate olfactory bulbs. The antennal lobes and olfactory bulbs receive projections directory from the olfactory receptor neurons located on the antenna and the olfactory epithelium of the nasal cavity, respectively. This places MB neurons third in order in the processing of the olfactory information in the insect brain. Similarly, olfactory bulb neurons of the vertebrate brain project to the primary olfactory cortex, piriform cortex, amygdala, which are important to olfactory information processing and emotional learning (Stoykova and Gross, 1994).

In addition to the characteristic similarities in neural pathways, molecular mechanisms controlling the development of comparable olfactory centers are of certain commonalities between arthropod and vertebrate. In vertebrate, *Pax6*, vertebrate counterpart gene of the *Drosophila eyeless*, is expressed in various regions of forebrain including the anlagen of the olfactory bulb, piriform cortex, and amygdala (Stoykova and Gross, 1994). Mutations of Pax6 result in profound defects in these forebrain structures

as well as other telencephalon regions (Stoykova *et al.*, 1996). Intriguingly, a mouse *dac* homolog is also expressed in the developing telencephalon in overlapping regions with the *Pax6* gene (Caubit *et al.*, 1999; Davis *et al.*, 1999; Kozmic *et al.*, 1999). The findings that, in both *Drosophila* and mouse, homologs of *Pax6* genes are expressed in and required for the development of the neural structures that are important to the olfactory perception and learning raises the possibility that these structures arose very early in brain evolution.

The question that how different type neurons are orderly generated and allocated to different topographical subdivisions is enormously important for developmental neuroscience. I anticipate that studies of the MB development in the *Drosophila* brain will lead to important insights into the molecular mechanisms controlling the sequential generation of neurons and their positioning into layers during the development of both vertebrate and invertebrate brains.