

## INTRODUCTION

Modern neuroscience has revealed that higher-order behaviors of brain such as perception, emotion and learning are closely associated with the precise wiring and physiological activity of the brain. Investigations into the developmental mechanisms that underlie the construction of the brain are one of the most challenging problems in neuroscience. For this challenge, elucidation of the cellular and molecular genetic mechanisms of brain development has been and still are problematic in vertebrate neural systems due to the billions of neurons involved. By contrast, containing significantly fewer neurons than the brains of vertebrates, *Drosophila melanogaster* offers a unique and promising opportunity to investigate the developmental mechanisms of the brain. With as few as 200,000 neurons (Strausfeld, 1976), *Drosophila* display an enormous range of organized behaviors including olfactory learning and cognition. Moreover, recent advances in developmental biology and sophisticated genetics in *Drosophila* open up an opportunity to utilize the fruit flies as a powerful model system for pursuing the molecular genetic mechanisms of brain development.

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). Behavioural studies imply that MBs are centers for higher-order functions including olfactory learning (for reviews see 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). Moreover, genes involved in the cAMP signaling system; *dunce*, *rutabaga*, *DCO* are preferentially expressed in the MBs and required for the olfactory learning and memory formation (Nighorn *et al.*, 1991, Han *et al.*, 1992,

Skoulakis *et al.*, 1993). These results strongly identify MBs as the principal sites mediating olfactory learning through the cAMP second messenger pathway.

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,  $\alpha$  and  $\alpha'$ , and the medial lobe is composed of three parallel lobes,  $\beta$ ,  $\beta'$  and  $\gamma$  (Crittenden *et al.*, 1998).

Developmental studies show that Kenyon cells are produced by the division of the four MB neuroblasts (Fig. 2), which are born at the 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). In general, neuroblasts are lying just beneath the outer surface of the cortex. Each neuroblast undergoes several asymmetric cell divisions to generate a specific set of unique intermediate precursor cells called ganglion mother cells (GMCs). Each GMC then divides symmetrically into two postmitotic neurons. Just after this division, neurons send fibers into the neuropile as they mature. Systematic clonal analysis has demonstrated that a single MB neuroblast sequentially generates three types of distinct MB neurons (Lee *et al.*, 1999). Neurons projecting into the  $\gamma$  lobe of the adult MBs are born first prior to the mid third instar larval stage. Subsequently, neurons projecting into the  $\alpha'$  and  $\beta'$  lobes are produced at the late third instar stage, and neurons projecting into

the  $\alpha$  and  $\beta$  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.

In the first part of this study, by high-resolution neuroanatomical techniques, I describe the early development of the embryonic MBs primordia and followed their axogenesis upto late embryonic stages. In the mid to late embryonic stages, the pioneer MB tracts extend along Fasciclin II (FAS II)-expressing cells and form the primordia for the peduncle and the medial lobe.

In the second part, I investigated transcriptional genes regulating the MBs development. In order to understand the cellular and genetic processes that control the development of MBs, I searched for putative regulatory factors, and found that the *Drosophila Pax6* genes, *eyeless* (*ey*; Quiring *et al.*, 1994; Halder *et al.*, 1995) and *twin of eyeless* (*toy*; Czerny *et al.*, 1999), and nuclear factor, *dachshund* (*dac*, Mardon *et al.*, 1994; Shen and Mardon, 1997) are expressed in the embryonic MB primordia. Previous studies have reported that *ey*, *toy* and *dac* have important functions in eye development. Whereas regulatory functions of *Pax6* homologs in eye development are evolutionally conserved (Callaerts *et al.*, 1997; Gehring and Ikeo, 1999), studies in vertebrates have shown that *Pax6* genes play important roles in brain development (Callaerts *et al.*, 1997; Stoykova and Gruss, 1996; Hanson and Heyningen, 1995; Stoykova *et al.*, 1996). The mouse *Pax6* gene is expressed in the telencephalon anlage, and mutations of *Pax6* result in profound defects in many of the forebrain structures including the olfactory cortex. In *Drosophila*, as do the vertebrate homologs, both *ey* and *toy* are expressed in the embryonic brain (Quiring *et al.*, 1994; Czerny *et al.*, 1999) but the neuroanatomical identities of the expressing cells and the functions of the two genes in brain development were unknown.

In this study, I showed that mutations of *ey* completely disrupted the neuropil structures of the MBs and a null mutation of *dac* resulted in marked disruption and aberrant projections of MB axons. Furthermore, genetic analyses demonstrated that, whereas *ey* and *dac* synergistically control the structural development of the MBs, the two genes are independently regulated in the course of MB development. These data argue for a distinct combinatorial code of regulatory genes for MBs as compared with eye development and suggest conserved roles of *Pax6* homologs in the genetic programs of the olfactory learning centers of complex brains.

In the third part, I performed high-resolution neuroanatomical studies of post-embryonic MBs of the *Drosophila* brain using various axonal and cell type specific markers. I described formation of discrete concentric layers in the larval peduncle and lobes as topological projections of concentric disto-proximal subdivisions of the Kenyon cells. FAS II 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* 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.