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Postembryonic Development of the Mushroom Bodies in the Ant, *Camponotus japonicus*

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ABSTRACT—Mushroom bodies (MB) are insect brain centers involved in learning and other complex behaviors and they are particularly large in ants. We describe the larval and pupal development of the MB in the carpenter ant, *Camponotus japonicus*. Based on morphological cues, we characterized the stages of preimaginal development of worker ants. We then describe morphological changes and neurogenesis underlying the MB development. Kenyon cells are produced in a proliferation cluster formed by symmetrical division of MB neuroblasts. While the duration of larval instars shows great individual variation, MB neuroblasts increase in number in each successive larval instar. The number of neuroblasts increases further during prepupal stages and peaks during early pupal stages. It decreases rapidly, and then neurogenesis generally ceases during the mid pupal stage (P4). In contrast to the larval period, the MB development of individuals is highly synchronized with physical time throughout metamorphosis. We show that carpenter ants (*C. japonicus*) have approximately half as many MB neuroblasts than are found in the honey bee *Apis mellifera*. Mature MBs of carpenter ants and honey bees reportedly comprise almost the same number of neurons. We therefore suggest that the MB neuroblasts in *C. japonicus* divide more often in order to produce a final number of MB neurons similar to that of honey bees.

Key words: brain, neuroblast, neurogenesis, social behavior, insect

INTRODUCTION

The insect mushroom bodies (MB) are paired brain compartments situated in the dorsal protocerebrum. The MB is a center of multimodal sensory integration involved in behavioral modulation. Functional studies show that the MB is involved in olfactory learning and memory (Davis, 1996; Heisenberg, 1998), consolidation of short-term and long-term associative memories (McBride *et al*., 1999), and visual context generalization (Liu *et al*., 1999), but is not required for direct visual or tactile learning (deBelle and Heisenberg, 1994; Wolf *et al*., 1998; Liu *et al*., 1999) in *Drosophila melanogaster*.

The general design of MBs, including the internal circuitry and connections with other brain regions, is highly conserved in all insects. The MB consists of a population of intrinsic neurons, referred to as Kenyon cells, whose somata occur in the cortex lying dorsally to the MB. The axons of Kenyon cells all run in parallel, thus forming a dense structure called the peduncle. Distally, the peduncle divides into two or more major subunits, referred to as the vertical and medial lobes, which are major output regions of the system. Most MB efferents project into the protocerebral neuropile surrounding the MB, from which they also receive some input (Ito *et al*., 1998). Kenyon cells have their dendrites organized within a dense neuropile referred to as MB calyx which is situated around the proximal segment of the peduncle. In most insect families, the calyx receives direct olfactory input from the antennal lobes and has therefore often been perceived as a secondary olfactory center. Unlike flies (*e.g. Drosophila*) and most other insect families, in Hymenoptera (wasps, bees and ants), the MB calyx also receives a substantial amount of direct visual input. In honey bees, paper wasps and many ants, the calyx is subdivided into three sub-compartments (Mobbs 1982; Gronenberg 1999; Strausfeld 2002). Olfactory projection neurons from the antennal lobes project to the calyx' lip region while visual fiber tracts originating in the optic lobes (medulla and lobula) innervate the collar region of the calyces. The collar also receives input from the mouthparts (probably gustatory; Durst *et al*., 1994). A third calycal region, the basal ring, receives segregated visual, antennal and probably other sensory input. In addition to sensory input, the calyx also receives efferents from the MB lobes, referred to as feed-

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back neurons (Gronenberg, 1987; Grünewald, 1999).

In contrast to the compartmentalized sensory input to the calyx, the separation between sensory modalities may be abolished at the MB output level by the prolific interconnection of the MB with surrounding neuropiles. The complex recurrent network formed by the MBs and the surrounding protocerebrum is thought to be capable of integrating and associating information of different sensory modalities (Mauelshagen, 1993; Rybak and Menzel, 1993).

The development of the MB has been most extensively investigated in *Drosophila*. Although the brain is significantly larger than the segmental ganglia and contains a variety of complex neuropiles, many brain neurons in *Drosophila* are derived through asymmetric neuroblast divisions, just as in the segmental ganglia (*cf.* Truman, 1990). Neuroblasts divide in an asymmetrical fashion to produce a new neuroblast and a smaller daughter cell, referred to as ganglion mother cell (GMC). A subsequent single division of each GMC generates two postmitotic ganglion cells that begin the differentiation process. In *Drosophila*, the MBs are created in their entirety by four neuroblasts per hemisphere. These neuroblasts divide continuously from early embryogenesis until late in the pupal stage (Ito and Hotta, 1992). The progeny of each neuroblast contribute to all parts of the mature MBs.

In contrast to the similarity of the basic design, the morphology of MBs differs significantly among insect taxa. In advanced Hymenoptera, the MBs are large and comprise a large number of Kenyon cells. The hymenopteran calyx is enlarged and cup-shaped and the interior of the calyx cup contains a large part of the Kenyon cell bodies, which also surround the outside of the cup. This highly developed sensory integration center correlates with a particularly large repertory of complex and plastic behaviors in social Hymenoptera, and it is generally assumed that the large mushroom bodies support the behavioral plasticity found in social Hymenoptera.

Current ideas about MB development of social insects are almost exclusively based on the honey bee. During the early MB development of honey bees, neuroblasts divide symmetrically, resulting in a duplication of neuroblasts (Malun, 1998; Farris *et al*., 1999). This mechanism dramatically increases the number of neuroblasts compared to the situation found in *Drosophila*. The dividing neuroblasts form neuroblast clusters. Simultaneously with the symmetric divisions, asymmetric divisions occur that produce GMC while regenerating the neuroblast. During later developmental stages, asymmetric cell division becomes more prevalent. Similar symmetric neuroblast divisions have also been observed in the optic lobes of *Manduca sexta* (Monsma and Booker, 1996; Champlin and Truman, 1998). The presence of a specialized region of aggregated neuroblasts characterized by symmetrical cell division allows producing many more cells than are produced in other brain regions.

Similarly to honey bees, all ant species show complex social organization that is controlled to a large extent by pheromones, many of which have been well characterized, both chemically and with respect to the specific behaviors they elicit. The social system and the basis for nestmate recognition in carpenter ants (*Camponotus*) has been well studied (Carlin and Hölldobler 1986, 1987, 1988; Hara, 2002). Odor learning and memory required for nestmate recognition can be manipulated experimentally in a predictable way (Carlin *et al*., 1987; Hara, 2003) and the MBs are thought to be involved in this behavioral plasticity. Little is known about the neuronal control of social behavior in insects. As the MB is known to be involved in the control of complex behaviors such as olfactory learning [*Drosophila* (deBelle and Heisenberg 1994; Heisenberg 1998; Dubnau *et al*., 2001); honey bee (Erber *et al*., 1980; Komischke *et al*., 2005)], foraging [honey bee (Withers *et al*., 1993, Durst *et al*., 1994, Farris *et al*., 2001), ants (Bernstein and Bernstein 1969, Gronenberg *et al*., 1996)] or place memory (cockroach; Mizunami *et al*., 1998), it is the prime candidate for brain structures underlying social behavior. We think that interspecific comparison of MB structure and development across social insects, and also between social and solitary insects, will help to better understand the evolution of social behaviors. The current study is a first step in this direction.

Like other holometabolic insects, *Camponotus* undergoes three different developmental phases (larva, pupa and adult), separated by metamorphic molts. A detailed characterization of these developmental phases is the basis for any studies of brain development but is not available for *Camponotus* spp. We therefore first provide a detailed description of the postembryonic development of the carpenter ant *Camponotus japonicus*, in order to provide a framework of developmental stages. We then describe a complete timeline of development of the ants' mushroom bodies from larval hatching to adult eclosion.

MATERIALS AND METHODS

Animals

Founding queens of *Camponotus japonicus* were collected in Tokyo, Japan. They were kept in plaster boxes under dim light conditions at 25°C and 60% relative humidity (*cf.* Hara, 2002). In *Camponotus japonicus*, as in most ant species, two kinds of brood development occur during the annual cycle, *i.e.* the fast brood and the slow brood (*cf.* Hölldobler and Wilson, 1990). Many larvae that hatch early in the season are able to complete development by the end of the summer (the fast brood). Broods hatching late in the season (fall in most cases) persist as larvae through the winter (the slow brood). In this study we focus on 'fast brood', all of which become workers under laboratory condition and are easily distinguished from sexuals as they do not have ocelli in *C. japonicus*.

Eggs were monitored every 12 hours in order to determine the precise moment of hatching. Shortly after hatching the larvae were marked individually with threads of different colors drawn from a surgical adhesive (AlonAlfa, TOAGOSEI), in order to identify individuals and monitor their molts. The larvae in feeding stages were maintained in their original colonies. The characters of each larval instar were standardized based on the observations of those 46 larvae that eventually eclosed as normal adults. For histological studies the larvae of every instar were collected within 12 hours after

Table 1. Larval instars in *Camponotus japonicus*

Instar	Duration (days) min – max	Head width (mm)		Body length (mm)	
		Mean±SD	min – max	Mean±SD	min – max
L1	1 – 6	0.407±0.0136	0.375 – 0.425	1.838±0.0983	1.625 – 2.075
L2	2 – 50	0.461±0.0164	0.450 – 0.500	2.173±0.2114	1.800 – 2.675
L3	2 – 20	0.541±0.0156	0.525 – 0.575	3.000±0.4145	2.125 – 3.750
L4	3 – 49	0.613±0.0181	0.600 – 0.675	4.129±0.5007	2.500 – 5.000

The development of 46 larvae was continuously tracked from hatching to adult eclosion.

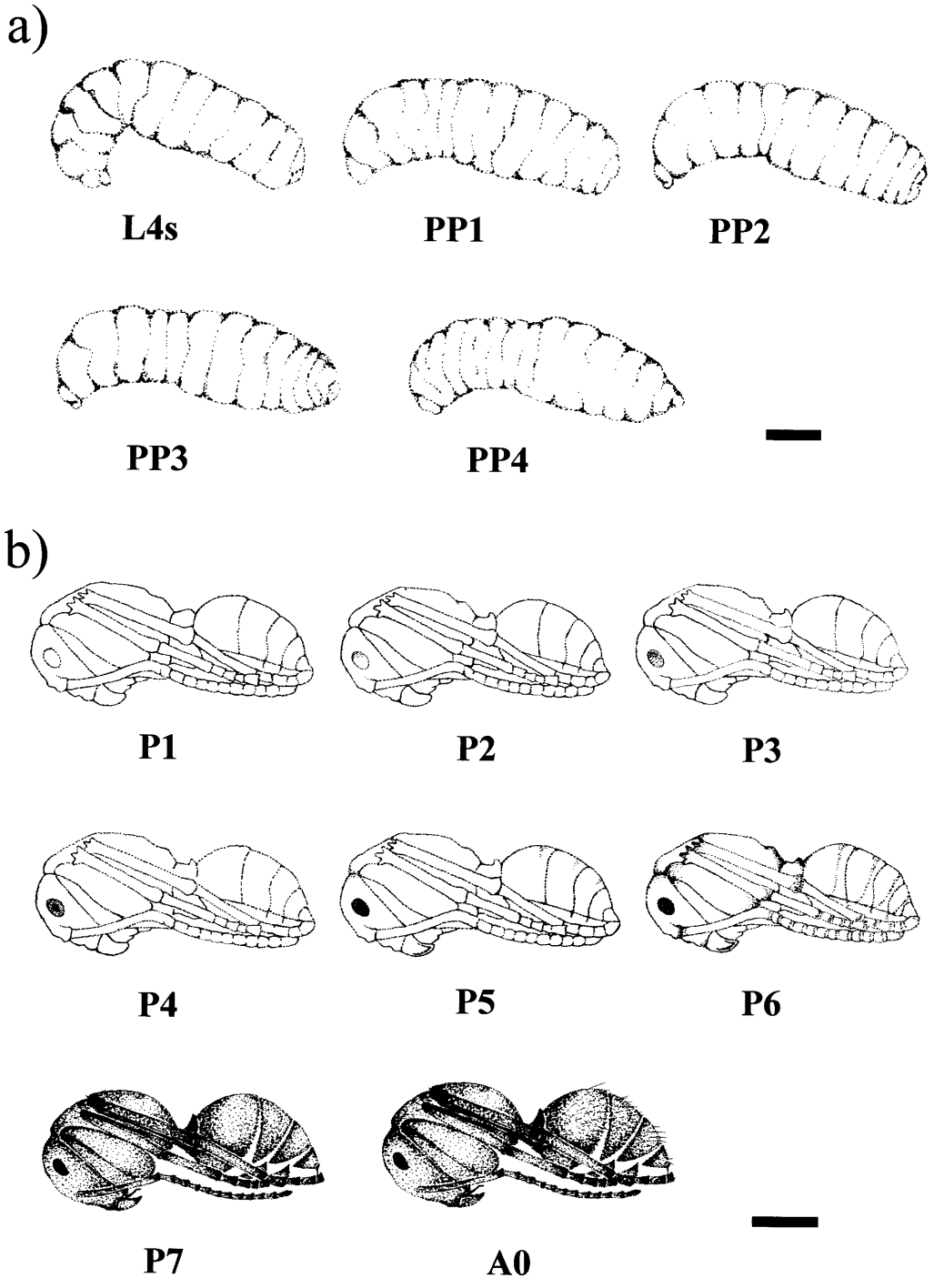

Fig. 1. Illustrations showing the morphological aspects of *C. japonicus* prepupal (a) and pupal (b) stages. Characteristics of each stage are detailed in Table 2. Scale bars=2 mm.

molting.

Cocoon-spinning larvae (L4s), prepupae and pupae removed from the cocoon were incubated individually in a culture dish at 25°C and 60% relative humidity. The prepupal and pupal stages were characterized based on observations of 74 larvae that eventually molted as adults. Body sizes were measured using a stereomicroscope (Stemi 2000, ZEISS).

Histological preparations and BrdU incorporation

For histological analysis the brains were dissected in phosphate buffered saline (PBS) and then fixed overnight in Bouin's solution. Thereafter, brains were rinsed, dehydrate in ethyl alcohol and embedded in paraffin. All samples were sectioned at 7 μm and stained with Delafield's hematoxylin and 1% eosin.

To visualize the dividing cells, BrdU (Amersham) was injected directly into the larval body at L1 ~ L3, or into the head region of L4 larvae, prepupae and pupae (approximately 0.3 μl of a 25 mg/ml BrdU/saline solution per animal). To describe the spatial and temporal aspects of cell proliferation, we observed the labeled profiles after 8 hours of BrdU incorporation at each stage. Generally, the number of labeled profiles corresponds to the number of BrdU-labelled nuclei, which, in turn, depends on the total number of proliferating cells, on the rate of proliferation, and on the duration of the cell cycle's S phase, during which BrdU is incorporated into the cell's DNA. If the treatment time is longer than the duration of the cell cycle, the number of labeled cells is independent of the duration of the S phase and reflects both the total number of proliferating cells and the rate of their proliferation. In *Drosophila*, the cell cycle duration of brain neuroblasts has been estimated to be about 1–2 hours during the active phase of neurogenesis (Truman and Bate, 1988; Ito and Hotta, 1992), and in MBs the cell cycle duration did not exceed 6 hours at the end of neurogenesis (Ito and Hotta, 1992). Therefore, we determined the number of labeled profiles after 8 hours of BrdU treatment to characterize the proliferation activity in the ant brain.

Brains treated with BrdU for 8 hours were dissected, fixed, and embedded as described above for histology, then sectioned at 7 μm. The immunostaining of incorporated BrdU was performed as described by Hara (2003).

Sections were viewed by brightfield light microscopy (Olympus BH-2). To avoid the observation errors, especially in counting the cell number, the digital images were used to analyze the serial sections of a brain. The digital images of each section were taken with the digital camera DP70 (Olympus), and aligned and merged for every brain using Adobe Photoshop 5.5.

RESULTS

After hatching from the egg, ants go through separate developmental stages that are described in the following two sections: larva, prepupa, pupa and adult.

Characterization of larval instars

The presence of four larval instars was confirmed in *Camponotus japonicus*. The duration of the respective instars varied strongly among individuals, hence chronological age could not be used for staging (Table 1). The body

Table 2. Prepupal and Pupal stages in *Camponotus japonicus*[a]

Stage[b]	Duration[c] (days) min – max	characteristics
L4s	0.5 – 1	Cocoon-spinning phase. Larva is rolled up. It becomes motionless.
PP1	1 – 1.5	Quiescent phase. Larva becomes unrolled. Internal: Newly forming cuticle begins to cover the brain. Antennal discs start to elongate.
PP2	0.5 – 1	Apolysis begins in the head region. Internal: Contents of an intestine pushed down to the rectum. Pupal head is distinct. Cuticle formation extends to the thorax and abdomen.
PP3	2 – 3	Apolysis extends to the thorax. Contents of rectum are excreted. Internal: Pupal thorax and abdomen are distinct. Antennae and legs are distinctly segmented yet short.
PP4	0.5 – 1	Apolysis reaches abdomen. Head and thorax appendages visible through larval cuticle. Internal: Antennae and legs elongate to almost final size.
P1	1 – 1.5	Pupal ecdysis. White compounded eyes and body.
P2	3 – 4	Light brown pigmentation of upper ommatidia sequentially progresses across compound eye (primary eye pigmentation). Body is white.
P3	2 – 4	Primary pigmentation covers entire compound eye; eye is light brown. Unpigmented ommatidia between pigmented ones start coloring (secondary eye pigmentation). Body is white.
P4	4 – 6	Secondary pigmentation wave reaches entire compounded eye; eye is brown. Another pigmentation wave starts at center of compounded eye (tertiary eye pigmentation). Body is white.
P5	2 – 3	Abdominal pigmentation starts; the striping of pigmentation progresses from dorsal to ventral. Antenna is white. Eye is dark brown.
P6	3 – 5	Antenna starts coloring. The striping of abdominal pigmentation reaches to ventral, and then unpigmented space between stripes starts coloring.
P7	1 – 2	Abdominal and antennal pigmentation are complete. Both dark brown.
A0	not identified[#]	A thin outer skin molts, allowing the antenna and legs to expand. Antennal segments formed adult type. Abdominal sensillae revealed. Imago still in cocoon. Voluntary movement of legs.

[a]: The normal development of the 74 larvae were continuously observed from L4s to adult molt. [b]: L4s=4th larval instar after cocoon spinning; PP1 ~ PP4=prepupal periods; P1 ~ P7=pupal periods; A0=adult still in the cocoon. [c]: minimum and maximum durations in each stage. [#]: The timing of eclosion from the cocoon are highly variable, which might depend on colony condition.

length differed significantly (one-way ANOVA, $p<0.01$) between larval instars (Table 1), but differed too much within each larval stage to be used as an indicator. In contrast, the head capsule width never overlapped among different instars (Table 1). While larvae were growing during each stage (increased body length), head width did not change for a given individual during a particular larval stage. Therefore, head width correlates tightly with and is the best indicator for larval stage.

Definition of metamorphic stages

The cocoon-spinning marks the beginning of the late fourth larval stage (L4s). The head width of L4s larvae (mean ±SD=0.6177±0.181 mm, minimum=0.600 mm, maximum =0.675 mm) was not significantly different from L4 (t-test, $p>0.01$), although the body length was considerably larger (6.144±0.3325 mm, minimum=5.667 mm, maximum=6.900 mm) than at the beginning of the fourth instar (4.129±0.5007 mm; Table 1). Movement of L4s larvae gradually ceases and the insect turns into an immobile prepupa. Approximately 5 days later, pupal ecdysis occurs. The pupa undergoes a series of changes in the pigmentation of the compound eyes and of the body. The pupal/imago molt occurs at the end of this phase. The changes of appearance during metamorphosis are illustrated in Fig. 1.

We divided the prepupal phase into four stages (from PP1 to PP4) according to the progression of apolysis from the cephalic to the posterior region. Formation of the imaginal cuticle inside the larval cuticle was also used to characterize prepupal stages. Seven pupal stages (from P1 to P7) were identified during pupal phase based on the pigmentation of eyes, abdomen and antennae. The imago in a cocoon (A0) forms a stage different from that of the newly emerged adult (A1), because social contact is an important event for social insects and may influence brain development during and immediately after eclosion (worker ants help their younger sisters to eclose from the cocoon). General aspects and the duration of each stage are summarized

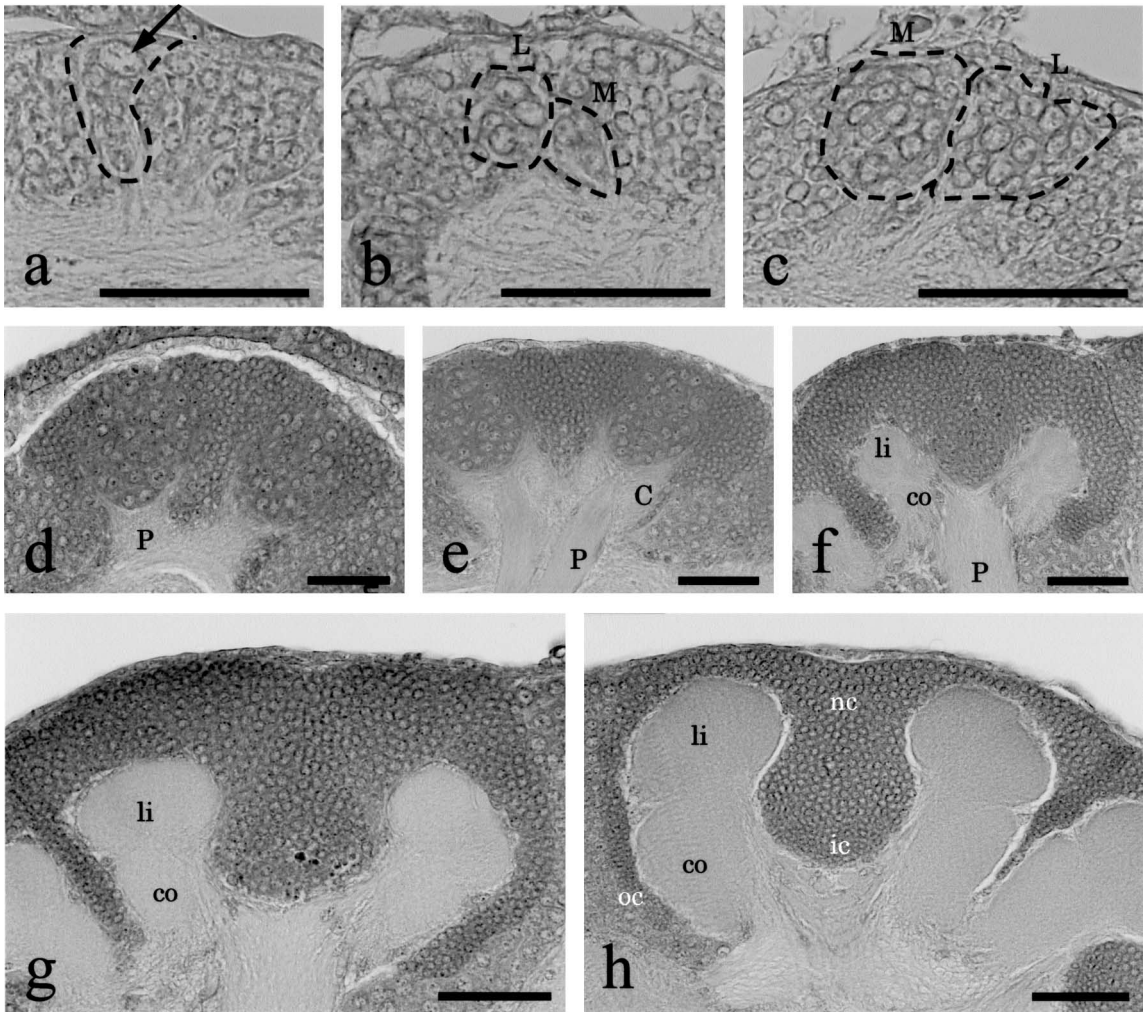

Fig. 2. Development of the mushroom bodies in *C. japonicus*. Aspects of the dorsal protocerebrum in larvae (L1; a, L2; b, L3; c), spinning larva (L4s; d), prepupa (PP2; e), pupae (P3; f, P5; g) and adult (A0; h). A solitary neuroblast (arrow) and its progeny are outlined in (a). Medial (M) and lateral (L) neuroblast clusters are outlined in (b) and (c). C, calyx; P, peduncle; li, lip; co, collar; ic, inner compact cells; nc, noncompact cells; oc, outer compact cells. Scale bars=50 μm.

in Table 2. Among all pupal and imaginal stages, there are no significant difference of body length (mean±SD =6.21± 0.29 mm), head width (1.31±0.77 mm), distance between eyes (0.95±0.55 mm), length of scapes (1.87±0.13 mm) and total length of antennal flagella and pedicel (3.07 ±0.16 mm) (one-way ANOVA, p>0.01: sample sizes; P1=19, P2=27, P3=26, P4=29, P5=23, P6=23, P7=26, A0=8, A1=24).

On average, it takes *C. japonicus* worker ants 24 days to metamorphose from spinning the cocoon to the final molt. This time is much longer than the 11 days metamorphosis takes honey bee workers (*Apis mellifera*; Bertholf, 1925). In general, the development depends on climate conditions, and on the temperature in particular. In our experiments, ant worker development tended to be shorter at temperatures higher than 25°C; we found the shortest developmental times (20 days) at 30°C, a temperature that is higher than what *C. japonicus* experience under natural conditions.

Gross morphological changes of the mushroom bodies through postembryonic development

In the newly hatched larva (L1), approximately 30 solitary neuroblasts with a diameter 14 μm are located in the periphery of the protocerbrum (Fig. 2a). Many of these stem cells divide asymmetrically to produce chains of ganglion mother cells (GMC) with diameters of 6 μm and their daughter cells with diameters of 5 μm. Solitary neuroblasts are easily recognizable by their stereotyped position and large size, whereas ganglion mother cells and neurons arising from individual neuroblasts form distinctive columns. In the dorsal brain region of L1 ~ L2 larvae, cell clusters can be discriminated from the surrounding tissue by their tight packing (Fig. 2b). This particular cluster (Fig. 2b) consisted of a small number of cells with a uniform appearance and with diameters of 10 μm. They are smaller than the solitary neuroblasts in the periphery of the brain and are larger than their progeny. Their progeny surrounds the cluster but does not form columns. Because of their histological and cytological similarity with neuroblast clusters in the honey bee (see **DISCUSSION**), they will be referred to as mushroom body neuroblast (MB neuroblast). Throughout L1 and L2, there was individual variation in cluster development: 14.7% of L1 larvae already had two distinct neuroblast clusters in each brain hemisphere (n=34), whereas in 12% of the L2 larvae were still without any cluster (n=25). At L3, a lateral and a medial cluster can be clearly discriminated in all larvae (Fig. 2c). At this stage (L3), clusters are formed by approximately 30 neuroblasts, all clusters have almost the same volume and no neuroblast was observed outside the clusters. A thin precursor of the medial lobe was identifiable at the end of the L3 stage in protocerebral neuropile (not shown). At the beginning of L4, each MB cluster comprises approximately 70 neuroblasts. At this time, the pedunclar neuropiles are formed but calyces are not yet visible. During the late L4 and the L4s stages, the number of MB neuroblasts increases to approximately 200 neuroblasts per hemisphere (Fig. 2d). The number of MB neuroblasts correlated with the number of larval instars (one-way ANOVA, p<0.01; Fig. 3a).

The calyces can be discriminated by prepupal stage 2 (PP2; Fig. 2e). At this stage, the number of MB neuroblasts increases further and peaks at approximately 500 neuroblasts per hemisphere (Fig. 3b). The volume occupied by Kenyon cell bodies grows rapidly during the prepupal stages. Likewise, the developing pedunculus and lobe neuropiles increase further in volume by the time of pupal ecdysis (Fig. 2e).

During pupal stages P1 and P2 the volume of calycal neuropile increases further. During the third pupal stage (P3) the number of MB neuroblasts decreases rapidly and concomitantly the volume of the neuroblast clusters declines (Fig. 3b). At this stage, the lip and collar regions become visible (Fig. 2f). At P4, the former MB neuroblast clusters do not contain any more neuroblasts but still ganglion mother cells, and the clusters disappear completely by the end of this stage. At P5, lip and collar regions can be clearly distinguished in the calyx (Fig. 2g). By the adult stage (A0) the MB neuropiles have further increase in volume (Fig. 2h). We were not able to determine when the calyx' basal ring is formed, but this is due to the fact that the basal ring is diffi-

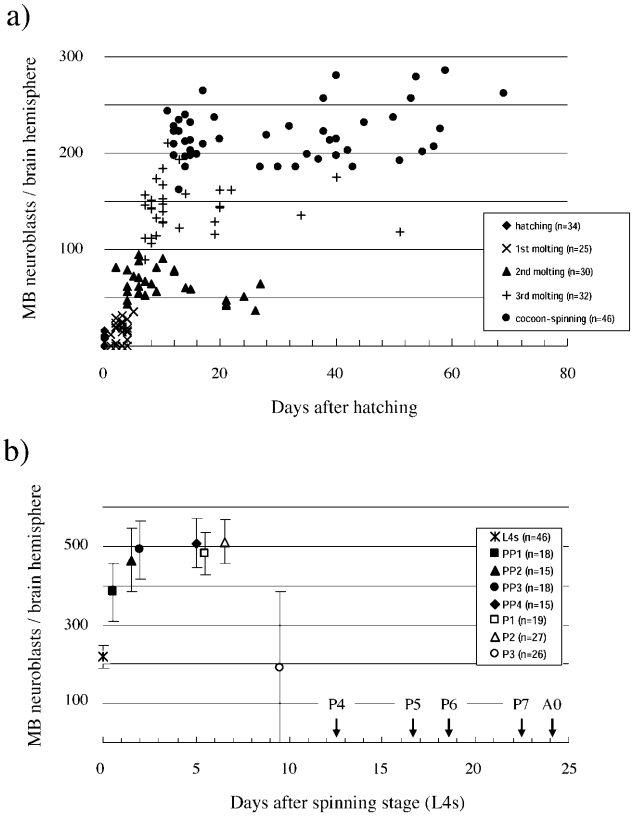

Fig. 3. Number of MB neuroblasts per hemisphere in larval (a) and prepupal ~ pupal (b) stages. (a) Each symbol represents one larva. There are significant differences among the classes (one-way ANOVA, p<0.01). (b) Each symbol represents mean value of one stage. Error bars represent SD. No neuroblast observed in the P4 (n=29), P5 (n=23), P6 (n=23), P7 (n=26), and A0 (n=8). Symbols and arrows point at the beginning of stages.

cult to discriminate even in mature ants.

Neurogenesis in the mushroom bodies

At L1, asymmetrical neuroblast mitoses were identified predominantly in the periphery of the brain (Fig. 4a). Throughout L1 to L4, few labeled cells with BrdU were observed in the MB cluster(s) (Fig. 4b). As mentioned above, we found an obvious increase in MB neuroblast number during these stages. Therefore, these patterns of BrdU incorporation reflect the low proliferation activity of MB neurobalsts (see **MATERIALS AND METHODS**). Their mitotic cycle might include long interphase and might not be synchronized with each other in the cluster.

At L4s, we observed more pronounced BrdU labeling in the MB clusters. In four out of six analyzed larvae (67%) approximately 60 neuroblasts were labeled per hemisphere by our 8-hour BrdU treatments at this stage (L4s). In contrast, in the other two larvae (33%), no MB neuroblasts were labelled but BrdU-labeled GMCs and new Kenyon cells were scattered throughout each cluster (Fig. 4c). In honey bees, three subpopulations of Kenyon cells can be discriminated in the adult brain based on their size and their posi-

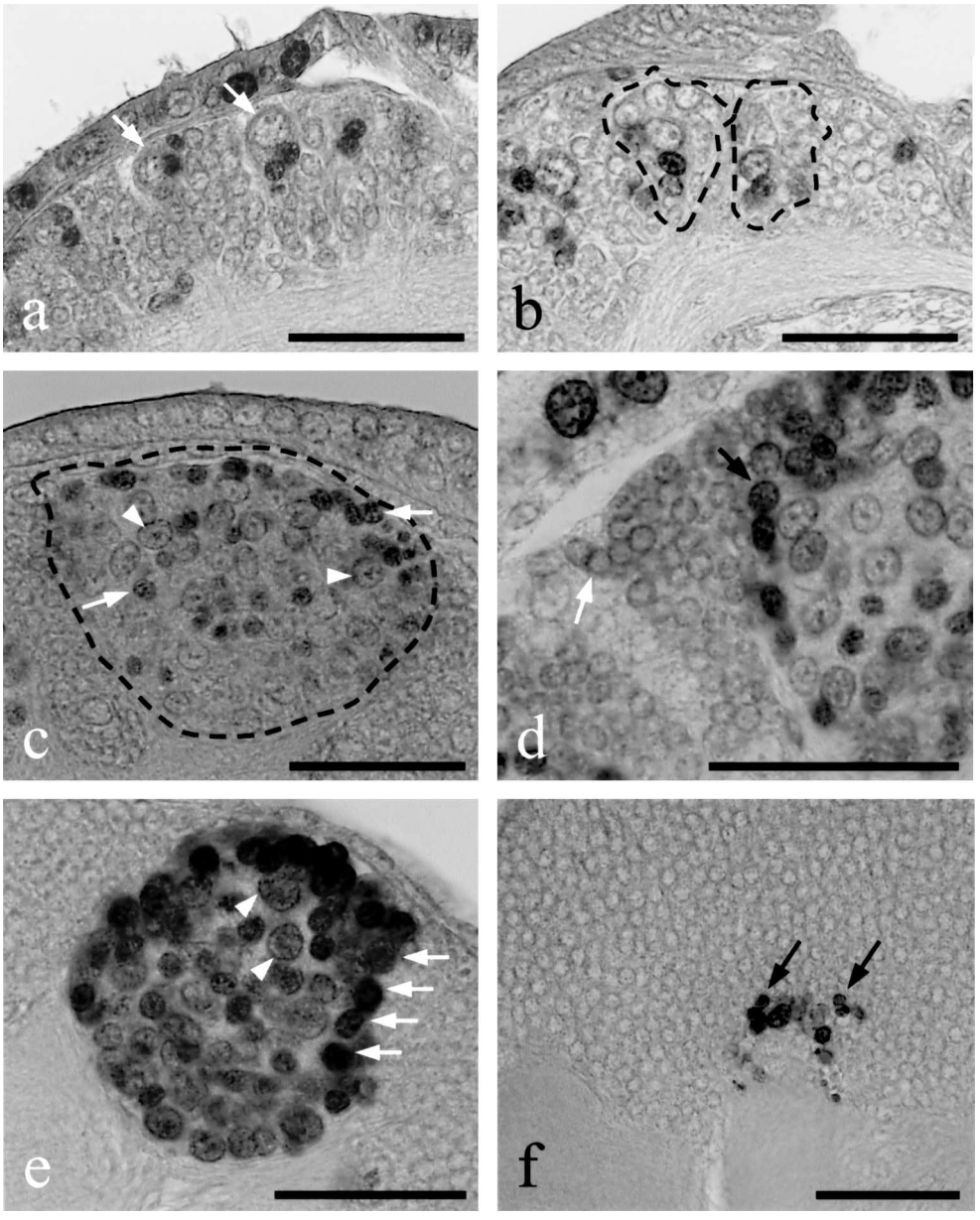

Fig. 4. Progressive changes of BrdU incorporation patterns during mushroom body development. (a) Dorsal protocerebrum regions in the L1 larva. White arrows indicate solitary neuroblast. (b) Mb neuroblast clusters (outlined) in the L2. (c) Neuroblast cluster (outlined) with ganglion mother cells (GMCs; white arrows) in the L4s. White arrowheads indicate MB neuroblast. (d) Higher magnification of the cluster boundary in the L4s. Note the cell size difference between labeled noncompact cells (black arrow) and non-labeled outer compact cells (white arrow). (e) Many labeled neuroblasts (white arrowheads) and GMCs (white arrows) are in the cluster of PP1. (f) Few labeled cells inside the calyx at the early of P4. Arrows indicate the labeled inner compact cells. Scale bars=50 μm.

tion with respect to the calyx: noncompact, inner compact and outer compact cells (Farris *et al*., 1999). We found similar Kenyon cell types in eclosing ant workers (Fig. 2h). In late larvae (L4s), the older neurons were clearly smaller than the more recently born cells, which were approximately 4 μm in diameter (Fig. 4d). Therefore, the first-born cells correspond to the outer compact cells of honey bees. We did not find notable difference in the number of BrdU-labeled neuroblasts and their progeny between in the lateral and medial clusters.

Mitotic activity peaked at PP1 ~ PP2 (Fig. 4e). BrdU was incorporated into approximately 300 MB neuroblasts per hemisphere within 8 hours. BrdU-labeled neuroblasts were present at the center of each cluster, which was lined by labeled GMCs (compare Fig. 4e and 4c). New Kenyon cells were pushed to the side of each neuroblast cluster and could thus be recognized as a thin sheath of small cell somata surrounding each neuroblast cluster. At PP3, the Kenyon cell population has increased substantially, so that a mass of tightly packed Kenyon cells of highly uniform size encircles each neuroblast cluster at this stage (not shown).

The number of BrdU-labeled neuroblasts remains very high until the second pupal stage (P2). By early P4 the proliferation of MB neuroblasts ceases abruptly from peak proliferation to zero (Fig. 3b). BrdU incorporation into both GMCs and Kenyon cells is observed in early P4. BrdU is also incorporated into the Kenyon cells that have smaller cell bodies (4 μm) than the non-labeled ones surrounding them (Fig. 4f). Therefore, these cells correspond to the inner compact cells of honey bees. From P5 onward, no BrdU-labeled cell was observed inside the cup.

Gliogenesis in the mushroom bodies

Although glia cells seem to proliferate in ways different from those of neurons, there is not yet sufficient evidence that the MB neuroblasts give rise to neurons exclusively. Newly produced glia cells are often hardly distinguishable from neurons based on their morphology. However, there is little doubt that the scattered BrdU-labeled cells surrounding neuropiles are glia cells, because within 8 hours neurons never migrate away far from the their sister cells. High levels of BrdU incorporation into the glia cells surrounding pedunclar neuropile were observed at the PP2 stage (Fig. 5a), and continued in the pupa until P2. Although the number of labeled glia cells subsequently decreased as Kenyon cell proliferation slowed in P3 (Fig. 5b), no BrdU labeling was observed in the glia cells situated around or inside the peduncles and lobes from P4 onward.

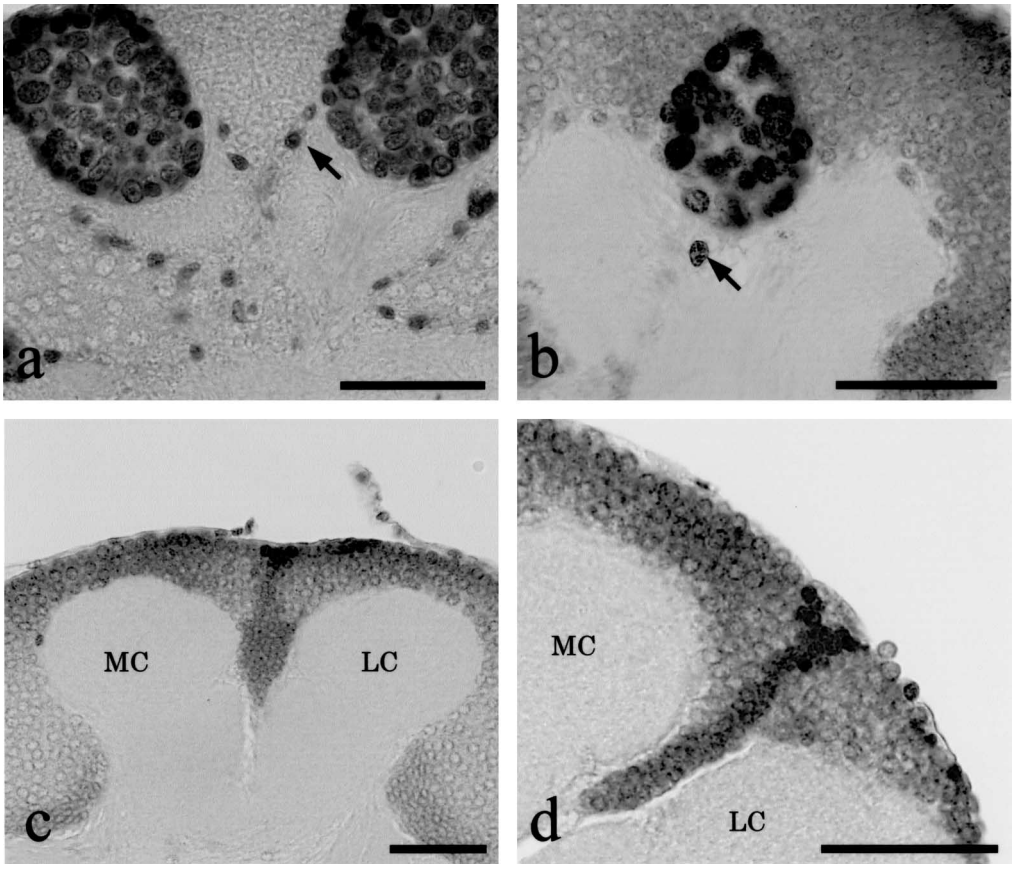

Fig. 5. Pattern of BrdU incorporations in mushroom body glial cells. (a), (b) During neurogenesis stages (PP2; a, P3; b), labeled glial cells were observed surrounding the calyx (arrow). (c), (d) In post-neurogenesis stages (P5; c, P7; d), mitoses were observed only in glia cells between lateral (LC) and medial (MC) calyces. Scale bars=50 μm.

At P5, a few small (4 μm) cells underneath the perineurium at the border between the medial and lateral Kenyon cell mass were clearly BrdU labeled (Fig. 5c). These are similar in size to the outer compact Kenyon cells, but they do not seem to be neurons because no neuroblast cell was found in their vicinity, rather, they appear to be derived from perineurium cells (Fig. 5d). BrdU incorporation into these cells continued in newly eclosed adults (four out of five A0 and twelve out of fifteen A1 adults).

DISCUSSION

We have characterized four larval instars phases and thirteen metamorphic stages in *Camponotus japonicus* workers, and we have described for each developmental step the neurogenesis and structural modifications that lead to the development of their mushroom bodies. As the MBs are involved in different aspects of social behavior (*e.g.* learning and memory, orientation; Strausfeld *et al*., 1998), a comparative view of the mushroom body development across Hymenopteran species might help to understand the variation of social behaviors in different species. At the present time, however, studies on the brain development of social insects are restricted to only a few species (honey bee: Malun, 1998; Farris *et al*., 1999; Leaf-cutting ant: Soares and Serrão, 2001). Therefore, it should be noted that this study provides significant information also in respect to the future studies.

Mushroom body neuroblasts in ants

The cluster of Kenyon cells precursors is one of the typical features in mushroom body development of *Camponotus japonicus*. Data gathered from both HE-stained and BrdU immuno-labeled sections revealed that the two cell clusters of each hemisphere persisted and showed mitotic activity until mid-pupal stages. Throughout larval and pupal development, the proliferation of Kenyon cell precursors in the clusters was never associated with the presence of large, asymmetrically dividing neuroblasts that are typically found in the periphery of the brain and that give rise to columns or rows of progeny. This finding together with the uniform size of the cells and the homogeneous distribution of BrdU-stained nuclei within the cluster strongly suggests that many Kenyon cell precursors undergo symmetrical cell divisions during larval and prepupal stages. These properties are very similar to those of MB neuroblasts in the honey bee (Malun, 1998; Farris *et al*., 1999). Therefore, social bees and ants have in common the organization and development of MB neuroblasts.

The neuroblast clusters are indispensable for producing a large number of neurons in a short time (Monsma and Booker, 1996; Champlin and Truman, 1998; Farrie *et al*., 1999). In *Camponotus ocreatus*, the mushroom bodies of adult workers have been estimated to be composed of approximately 130,000 Kenyon cells per hemisphere (Ehmer and Gronenberg, 2004). Considering that *C. ocreatus* and *C. japonicus* workers are of similar body, head and brain size (Ehmer and Gronenberg, 2004; Hara, 2003), we assume the number of Kenyon cells also to be similar in the two species. Hence in *Camponotus japonicus* ant workers, about 500 neuroblasts per hemisphere eventually produce approximately 130,000 Kenyon cells, suggesting that the neuroblasts have to divide about 130 times. For comparison, in honey bees, approximately 1,000 MB neurobalsts per hemisphere produce 170,000 Kenyon cells approximately 10 days (Farris *et al*., 1999). The cell cycle period of MB neuroblasts is between 1.1 and 1.5 hours in honey bees (Farris *et al*., 1999). Based on our study, it takes *C. japonicus* approximately two weeks, 1.4 times as long as honey bees, to produce the full set of Kenyon cells. Assuming that carpenter ants have a similar cell cycle period as honey bees, two weeks should suffice to generate the number of Kenyon cells found in adult MBs. During this time, MB neuroblasts in the ant have to undergo more cell division cycles than is required in honey bees.

The embryonic origin of the MB neuroblasts could not be determined in our study. In a few preparations, we were able to distinguish one cluster of MB neuroblasts per brain hemisphere in the first instar. The total number of the solitary neuroblasts appears to be rather constant at early postembryonic stages, even after the two neuroblast clusters supplying each MB have been formed. Therefore, the MB neuroblasts may be derived from a very small number of originally large neuroblasts, as has been shown in the butterfly *Danaus* (Nordlander and Edwards, 1970), and is assumed for honey bees (Malun, 1998).

Calyx development of the ant

The calyx is a major MB input region and receives prominent afferents from the antennal lobes and, in Hymenoptera, also from the optic lobes. In social Hymenoptera, the calyx neuropile is arranged into three concentric regions: lip, collar and basal ring. These compartments can be easily discriminated in honey bees and paper wasps, while the basal ring may be hard to discriminate in many ants (Mobbs, 1982; Ehmer and Hoy, 2000; Gronenberg, 2001). In the honeybee brain, the lip region receives olfactory input from the antennal lobes and contains dendrites of noncompact Kenyon cells. The collar receives visual input and contains noncompact Kenyon cell dendrites and dendrites of some outer compact Kenyon cells. The innermost basal ring receives segregated olfactory and visual input and is composed of dendritic arborizations arising from the inner and outer compact cell regions (Mobbs, 1982; Arnold *et al*., 1985; Strausfeld, 2002). The distribution of the compact and noncompact Kenyon cells in the honeybee mushroom bodies would most simply be accounted for by their pattern of neurogenesis. The outer compact cells, which are farthest from the center of each calyx, are born earliest and are pushed outward by the younger noncompact cells, which are in turn pushed away by the central inner compact cells. Neurons that are born first are probably also the first to

extend dendrites and to form their respective calyx compartments. Indeed, lip and collar arise first before the basal ring appears in honey bee (Farris *et al*., 1999).

We show that the sequential production of Kenyon cell populations in carpenter ants is very similar to that in honey bees. We were not able to identify when the basal ring arises. In many ant species the basal ring is smaller than it is in honey bees or paper wasps and may be difficult to discern when using general staining methods (*cf.* Ehmer and Gronenberg, 2004). We show that the mitotic activity, as indicated by BrdU incorporation, is continuing at the bottom of each calyx and generates inner compact cells at pupal stage P4, when the lip and collar are distinguishable. Comparable inner compact cells in honey bees contribute to basal ring formation at this developmental stage. Therefore, we conclude that the program for mushroom body development is mostly conserved between carpenter ants and honey bees.

The presence of Kenyon cells is essential for outgrowth of projection neuron axon collaterals into the calyces, although there is not a strict correspondence between the three Kenyon cell types and the division of the calyx neuropile (Malun *et al*., 2002). Presumably, the age gradient of Kenyon cells (*i.e.* their relative neural birthdate) contributes to target finding of projection neurons and to subdivisions of the calyces. In the honey bees, the processes of olfactory projection neurons and of visual projection neurons innervate strictly different neuropile regions, the lip and the collar, respectively, at an early pupal stage at which the calycal subdivisions are already identifiable (Schröter and Malun, 2000). We show that the sequential pattern of subdivision development in the ant calyx is conserved and is very similar to that found in honey bees. This suggests that during pupal stage P3, Kenyon cell dendrites would be actively be branching in the newly formed the lip and collar compartments, and the projection neurons from the antennal lobes and optic lobes would arborize in these regions.

In honey bees and *Drosophila*, the youngest Kenyon cells are expected to contribute most to adult plasticity, as the earlier-born cells have had more time before adult behavioral maturation to develop the full extent of their projections (Ito and Hotta, 1992; Farris *et al*., 1999). We show that in carpenter ants the duration of post-neurogenesis is approximately two weeks, which is three times longer than in the honey bee. This would suggest that in ant workers most of the Kenyon cells may have completed their process development and little potential for morphologic plasticity may be left by the time of adult eclosion. However, in honey bees and in carpenter ants morphological changes ocurr long after adult hatching. Such morphological changes result in differences in MB neuropile volume and are assumed to underlie behavioral plasticity such as found during the transition from nest worker to forager (Withers *et al*., 1993; Durst *et al*., 1994; Gronenberg *et al*., 1996).

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