Synaptic organization in the adult honey bee brain is influenced by brood-temperature control during pupal development

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Recent studies have shown that the behavioral performance of adult honey bees is influenced by the temperature experienced during pupal development. Here we explore whether there are temperature-mediated effects on the brain. We raised pupae at different constant temperatures between 29 and 37°C and performed neuroanatomical analyses of the adult brains. Analyses focused on sensory-input regions in the mushroom bodies, brain areas associated with higher-order processing such as learning and memory. Distinct synaptic complexes [microglomeruli (MG)] within the mushroom body calyces were visualized by using fluorophore-conjugated phalloidin and an antibody to synapsin. The numbers of MG were different in bees that had been raised at different temperatures, and these differences persisted after the first week of adult life. In the olfactory-input region (lip), MG numbers were highest in bees raised at the temperature normally maintained in brood cells (34.5°C) and significantly decreased in bees raised at 1°C below and above this norm. Interestingly, in the neighboring visual-input region (collar), MG numbers were less affected by temperature. We conclude that thermoregulatory control of brood rearing can generate area- and modality-specific effects on synaptic neuropils in the adult brain. We propose that resulting differences in the synaptic circuitry may affect neuronal plasticity and may underlie temperature-mediated effects on multimodal communication and learning.

In honey bee colonies, brood temperature is controlled precisely within a temperature range of 33–36°C (1, 2). In the central brood area, fluctuations are as small as 35 ± 0.5°C during the pupal period (3, 4). Exposure to strong deviations from normal brood temperatures is known to result in increased mortality and morphological deficits (1, 5). Environmentally induced temperature changes within the hive are compensated by individual honey bee workers via endothermic heat production or evaporation cooling (4, 6, 7). Thermoregulation during winter is achieved also by endothermic heat production, but with lower absolute temperatures and less precision compared with brood rearing in summer (8, 9). A recent study demonstrated that the temperature experienced during pupal development influences the behavioral performance of adult bees (10). Worker bees that had been raised at lower temperatures (within the range of naturally occurring temperatures) performed less well in dance communication and olfactory learning than bees raised at higher temperatures.

As in other holometabolous insects, postembryonic development in honey bees includes complete larval–adult metamorphosis. In the pupa, the larval nervous system becomes completely remodeled to accommodate the development of adult-specific sensory organs and motor systems, which are associated with the extraordinary changes in behavior. The hormonal and neuronal processes underlying this remarkable plasticity have been the subject of numerous studies, most of them performed on the sphinx moth (*Manduca sexta*) and *Drosophila* (reviewed in refs. 11–14). Neurometamorphosis includes extensive growth of neurons and glia, cell proliferation, apoptosis, cell migration, and synaptogenesis, all of which are likely to be affected by temperature either directly or indirectly [e.g., via effects on neurons, glia, neurosecretory cells and/or the hormonal system (14, 15)]. In contrast to general effects of temperature on embryonic growth or the duration of larval and pupal development, more specific effects on the maturation of the metamorphosing nervous system rarely have been investigated (16). Recently, a study of the development of the olfactory system in the brain of the moth *M. sexta* has shown that temperature gradients influence proper formation of the antennal lobes by affecting neuron–glia interactions and axon pathfinding (17). In lower vertebrates, temperature manipulations during embryonic development affect sex determination and adult aggressive behavior, most likely mediated by changes in sexually dimorphic brain nuclei (18, 19). In mammals, slight temperature increases during embryonic development were shown to have profound effects on the nervous system (20).

In the present study, we investigated whether small changes in the temperature normally maintained during pupal development of honey bees may influence the synaptic maturation in the developing nervous system. The results show that different rearing temperatures cause area- and modality-specific effects on synaptic complexes within the mushroom bodies (MBs), higher integration centers in the insect brain. Effects occurred within the range of temperatures normally maintained by brood-temperature control. Potential consequences of changes in the synaptic circuitry for neuronal plasticity and behavioral performance are discussed.

Materials and Methods

Animals and Temperature Treatment of the Pupae. Colonies of the European honey bee (*Apis mellifera carnica*) were used for the experiments. To synchronize brood, egg-laying queens were confined to single brood combs for 24 h by using wire-mesh cages that permitted passage of only workers. Shortly after brood-cell capping, combs were transferred into incubators (Bachofer 400 HY-E, Reutlingen, Germany; Rumed 1000-72039, Laatzen, Germany; Sanyo MIR-153, Bad Nenndorf, Germany). During the entire pupal phase, the temperature was set to constant temperatures at 28, 29, 30, 31, 32, 33.5, 34.5, 35, 36, 37, and 38°C (three to four brood combs with 400–1,100 capped brood cells at each temperature) and monitored continuously within brood cells by using fine thermoprobes (Almemo 2290-8 V5, Holzkirchen, Germany). Deviations from preset temperatures were less than ±0.2°C. For all temperature treatments, the duration of pupal development and the emergence rates were recorded.

Neuroanatomical Techniques and Immunocytochemistry. After emergence, immunofluorescence staining was performed with brains of worker bees 1 (total of 90) and 7 (total of 18) days

Abbreviations: MB, mushroom body; PN, projection neuron; KC, Kenyon cell; MG, microglomeruli.

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old. The latter were taken from the group of bees that had been tested in a conditioning paradigm in a previous study (10). Brains were dissected in cold physiological saline (130 mM NaCl/5 mM KCl/4 mM $MgCl_2$/5 mM $CaCl_2$/15 mM Hepes/25 mM glucose/160 mM sucrose, pH 7.2), immediately immersed in cold 4% paraformaldehyde in 0.1 M PBS, pH 7.2, fixed overnight at 4°C, and then washed three times in PBS. After embedding in 5% low-melting-point agarose (Agarose II, no. 210-815, Amresco, Solon, OH), brains were sectioned in a frontal plane (100 or 150 μm) with a vibrating microtome (Leica VT 1000S, Nussloch, Germany). Free-floating agarose sections were preincubated in PBS with 0.2% Triton X-100 and 2% normal goat serum (ICN, no. 191356) for 1 h at room temperature. To label neuronal F-actin, sections were incubated in 0.2 units of Alexa Fluor 488 phalloidin (Molecular Probes, A-12379) in PBS for 2 days at 4°C (21). For double-labeling experiments, preparations were incubated simultaneously with a monoclonal antibody against the *Drosophila* synaptic-vesicle-associated protein synapsin I (1:50; SYN-ORF1; kindly provided by E. Buchner, University of Würzburg, Würzburg, Germany) (22) and with phalloidin. After five rinses in PBS, double-labeled preparations were incubated in Alexa Fluor 568-conjugated goat anti-mouse secondary antibody (1:250; Molecular Probes, A-21124) in 1% normal goat serum/PBS for 2 h at room temperature to visualize synapsin.

Axonal projections of antennal-lobe projection neurons (PNs) and dendrites of Kenyon cells (KCs) were labeled with rhodamine dextran (Microruby, Molecular Probes, D-7162) (23). Tips of glass electrodes were coated with the dye and inserted into the antennal lobe neuropil or the KC soma cluster in freshly dissected brains. The dye was allowed to diffuse for ≈3 h. Brains were fixed and processed as described above.

To label cell nuclei, sections were incubated for 15 min in 25 μg/ml propidium iodide (Molecular Probes, A-11003) in PBS with 0.2% Triton X-100 at room temperature. Sections were finally washed in at least five changes of PBS, transferred into 60% glycerol/PBS for 30 min, and mounted on slides in 80% glycerol/PBS.

Laser-Scanning Confocal Microscopy, Image Processing, and Data Analysis. Preparations were viewed with a laser-scanning confocal microscope (Leica TCS SP). Optical sections were imaged at intervals of 0.6–5.0 μm. In double-labeled preparations, the two channels were merged with the use of pseudocolors. Image processing was performed with Zeiss IMAGE BROWSER and Corel PHOTOPAINT and DRAW (Corel, Ottawa) software. 3D reconstructions were carried out with AMIRA software (Indeed-Visual Concepts, Berlin). Statistical tests were performed with SPSS software (Chicago). Details about quantitative evaluation of neuroanatomical data are described in *Results*. For statistical analyses, data were evaluated by using one-way ANOVA (Kruskal–Wallis one-way ANOVA on ranks; $P < 0.05$). Results are presented as mean ± SD.

Results

Temperature Dependence of Emergence Rate and Duration of Pupal Development. The period of postembryonic development in honey bees includes 2 prepupal stages and 9 pupal stages (sealed or capped brood) (23, 24). Emergence rates were highest between 31 and 36°C (89–100%), drastically dropped at higher and lower temperatures, and came to zero at 28 and 38°C (Table 1), largely confirming previous studies (1, 5). Pupal development was shortest between 34.5 and 37°C (10–11 days), increased at lower temperatures, and went up to almost twice the normal duration at 29°C (19–22 days). After emergence, bees reared between 32 and 36°C exhibited no obvious morphological and/or behavioral deficits. Some of the bees reared at <32°C and >36°C, however, showed malformations of their wings, stinger, proboscis, or legs. Others had no obvious morphological defects. From these groups (29, 30, 31, and 37°C), only those bees with no apparent morphological defects were used for neuroanatomical analyses.

Table 1. Temperature dependence of honey bee postembryonic development

Rearing temperature, °C	Pupal development, days	Emergence rate, %
28	—	0
29	19–22	8–12
30	17–19	66–73
31	14–15	89–100
32	12–15	98–100
33.5	11–12	97–100
34.5	10–11	94–100
35	10–11	96–98
36	10–11	93–99
37	10–11	36–42
38	—	0

Duration of pupal development and emergence rates in honey bee workers raised at different temperatures (minima/maxima from observations on three to four brood combs for each temperature).

Immunofluorescence Labeling of Microglomeruli in the MB Calyx. Fluorophore-conjugated phalloidin, which binds specifically to F-actin (25, 26), labeled all known synaptic neuropils in the honey bee brain (Fig. 1) (21, 27). The MB calyx was among the most intensely labeled structures, and its three subdivisions (lip, collar, and basal ring) (28) could be identified easily (Figs. 1*B* and 2). At higher magnification, spheroidal structures of ≈3 μm in diameter became clearly visible (Fig. 1 *C–E* and *M*). These microglomeruli (MG) represent distinct synaptic complexes in the calyx neuropil, each comprising a central bouton from PN axons surrounded by many KC dendritic spines and processes from other extrinsic neurons (27, 29–31). Synapsin-IR, which is associated with synaptic vesicles, stained the central bouton of MG, whereas phalloidin labeled a ring-like surrounding region (Fig. 1 *C–E*). Labeling of antennal-lobe PN axons (Fig. 1 *F–J*) and KC dendrites (Fig. 1 *K* and *L*) combined with phalloidin labeling revealed that PN boutons occupy the central core of MG, and KC dendrites colocalize with phalloidinergic rings (Fig. *K–M*), indicating that F-actin is located predominantly in the dendritic compartments of MG.

Changes in MG Associated with the Temperature Experienced During Pupal Development. The MB-calyx lip receives primarily olfactory input, whereas the collar is innervated by PNs from the optic ganglia, and the basal ring receives input from both modalities (30). The number and density of MG in the calyx lip and collar of freshly emerged bees were estimated from optical sections at a defined plane in the central brain. At this plane, the MB lip and collar were sectioned transversely, and the pedunculi and two divisions of the central body complex were clearly visible (Fig. 1 *A* and *B*). Phalloidin-labeled MG profiles were counted in the inner branch of the lateral calyx and the outer branch of the medial calyx of both sides (Fig. 1*A*, 1–4). Counts were performed blindly by one person and compared with samples counted by another person. Cross-sectional areas were measured in the same optical section. Variations between repeated counts and counts by two individuals were <8%. The numbers of MG profiles in the four calyces of each brain were averaged, and a mean was calculated from four brains (16 calyces) at each temperature. In the collar, MG are arranged in two distinct subdivisions (Fig. 2 *Upper*): an outer region with densely packed

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Fig. 1. Immunofluorescence labeling of MG in the MB calyx (CA) of the honey bee brain. (*A*) Overview of the central brain labeled with phalloidin (green) and propidium iodide (red). (*B*) Higher magnification of MB calyces (BR, basal ring; CO, collar; LP, lip) and peduncle (PED). (*C–E*) Double labeling of the calyx lip (box shown in *B*: synapsin-IR, red; phalloidin, green). Merged images in *E* with *Inset* show an individual MG. (*F–J*) Antennal lobe PNs (*G*, red) and phalloidin (*H*, green); the image is merged in *F* and *J*. The arrow in *F* indicates PN axons. (*K* and *L*) KC dendrites (*K*, red; 16.2-μm stack of 27 sections) and phalloidin labeling (*L*, green). Merged images of single optical sections are shown in *L*; the arrows indicate MG and overlap. (*M*) 3D reconstruction of MG (synapsin-IR, red; phalloidin, green). AL, antennal lobe; CC, central complex. [Scale bars: *A*, 200 μm; *B*, 100 μm; *C–E*, 20 μm (*Inset*, 3 μm); *F*, 50 μm; *G–J*, 20 μm; *K*, 20 μm; *L*, 10 μm; and *M*, 3 μm.]

MG and an inner region with loosely arranged MG. MG numbers were estimated for three circular areas (20 μm in diameter) in the dense portion and extrapolated to the total dense area.

In the lip, the area and number of MG differed at different rearing temperatures (Fig. 2 *Lower Left*). Clear changes in the number of MG profiles were found at temperature differences of 1°C (e.g., 34.5 vs. 33.5°C, $n = 4$, $P < 0.001$; 34.5 vs. 35°C, $n = 4$, $P < 0.004$; 34.5 vs. 36°C, $n = 4$, $P < 0.001$). The number of MG profiles was highest at 34.5°C, decreased above and below, and

Fig. 2. Changes in MG with different rearing temperatures (1-day-old bees). (*Upper*) Branch of the MB calyx labeled with phalloidin (measured areas are indicated). (Scale bar, 50 μm.) (*Lower*) The top histogram shows changes in cross-sectional areas of the lip (*Left*) and collar [*Right*; white bars indicate the loose region (L), and gray bars indicate the dense region (D)]. The middle histograms show the number of MG profiles in the lip (*Left*) and dense portion of the collar (*Right*) (a and b indicate significance). The bottom histograms show estimated density of MG in the lip (*Left*) and dense region of the collar (*Right*) (normalized to 1,000 μm^2).

dropped to almost 50% at 29 and 37°C. Synapsin-IR indicated that the number of presynaptic boutons was also affected (data not shown). The density of MG was highest at 33.5–35°C and at 29°C. In the collar, effects were less obvious between 30 and 36°C (Fig. 2 *Lower Right*). A significant decrease in MG profiles was observed at 29 and 37°C (34.5 vs. 29°C, $n = 4$, $P < 0.001$; 34.5 vs. 37°C, $n = 4$, $P < 0.001$). The proportion of the dense (70–72%) and loose (28–30%) regions remained essentially constant.

The sizes of individual MG showed a tendency to increase at low and high temperatures. At 34.5°C in the lip, the outer diameter of MG profiles ranged around 3 μm (3.09 ± 0.2 μm; 60 randomly selected MG from 3 bees). At 29 and 37°C, MG with diameters >3.5–4 μm were observed frequently. MG profiles in the collar were smaller (1.89 ± 0.15 μm at 34.5°C; 60 MG from 3 bees), and temperature-dependent changes were not detectable.

Persistence of Changes in MG After the First Week of Adult Life. To determine whether changes in MG numbers persist, we performed similar measurements in the MB calyx of 7-day-old bees (Fig. 3). The counts of MG profiles yielded similar values to 1-day-old bees (34.5 vs. 32°C, $n = 4$, $P < 0.02$; 34.5 vs. 36°C, $n = 4$, $P < 0.03$), and absolute numbers and densities of MG were not different from those in freshly emerged bees. In the collar, as in 1-day-old bees, changes in MG were not detectable. The results indicate that the temperature-mediated ef-

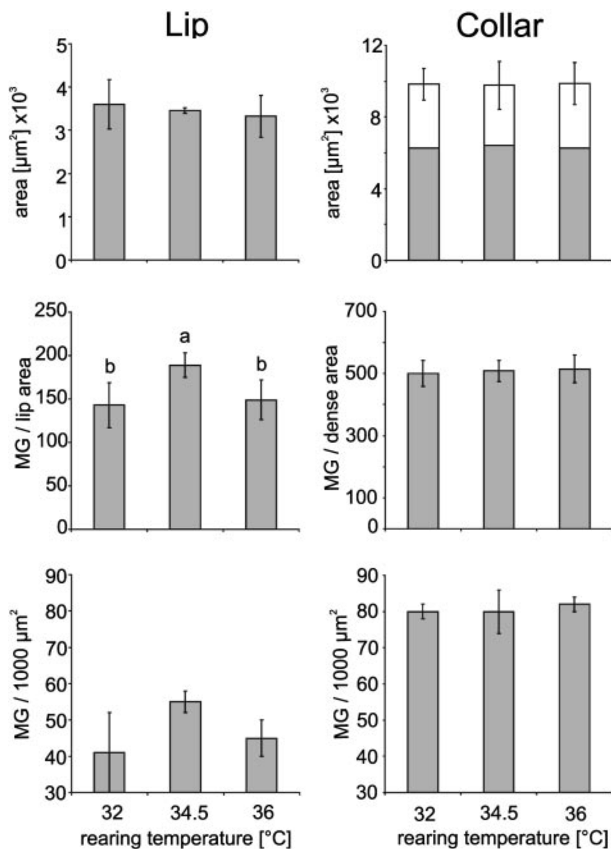

Fig. 3. Changes in MG with different rearing temperatures (7-day-old bees). (*Top*) Cross-sectional areas in the lip (LP, *Left*) and collar (CO, *Right*; white bars indicate the loose region, and gray bars indicate the dense region). (*Middle*) Number of MG profiles in the lip (*Left*) and the dense region of the collar (*Right*) (a and b indicate significance). (*Bottom*) Estimated density of MG in the lip (*Left*) and dense region of the collar (*Right*) (normalized to 1,000 μm^2).

fects on MG numbers persist at least during the first week of adult life.

Discussion

This study shows that brood-temperature control can influence synaptic organization in the brain of an adult honey bee. The most important finding is that temperature-mediated effects are position- and modality-specific even in adjacent areas of the MB calyx. Temperature-mediated changes occurred within the range of natural variations in brood temperature and persisted at least through the first week of adult life. The temperature-based plasticity in the synaptic circuitry may affect behavioral performance and the start and/or rate of behavioral transitions.

We chose the MB calyces for our analyses for three reasons. First, previous work suggests that the MBs are involved in higher-order computations such as learning and memory (e.g., refs. 32–37), which is important for complex behavioral tasks performed by honey bees. Second, the adult MBs express age- and experience-dependent volume changes (38–40), indicating that structural changes in the MBs are related to behavioral plasticity. Third, MG in the MB calyx represent distinct synaptic units (30, 31), which we were able to visualize and quantify at excellent resolution (21, 27).

The number of MG in the adult MBs changed with the temperature experienced during pupal development, which could be caused by presynaptic (PN boutons) and/or postsynaptic (KC dendrites) modifications. Previous studies have shown that neurogenesis is active until pupal stage four, decreases

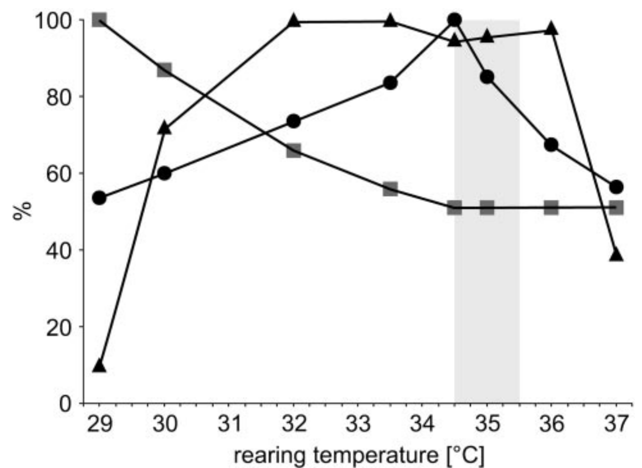

Fig. 4. Thermoregulation matches optimal developmental time, emergence rate, and synaptic-neuropil development (shown for the MB-calyx lip). Shortest development (rectangles), highest emergence rate (triangles), and highest number of MG in the lip (circles) overlap in the narrow range normally maintained by thermoregulation in central brood cells (gray area).

drastically after onset of apoptosis, and is absent in the adult brain (41, 42). The adult MBs exhibit age- and experience-related volume changes, as shown also for fruit flies, ants, and honey bees (39, 43–47). In the fruit fly, MB volume was affected by visual experience, and in ants and honey bees, volume increase was associated with age and behavioral maturation. In the bee and *Drosophila*, volume measurements in the antennal lobes also revealed age- and experience-related changes in distinct olfactory glomeruli (48, 49). In the honey bee, volume changes in the MBs were suggested to be caused mainly by dendritic growth of KCs (40). Changes in neuronal processes during experience-dependent maturation of the nervous system are common to many sensory systems. In the vertebrate visual system and olfactory bulb, excess dendritic branches are pruned during maturation (50, 51). Our results indicate that temperature-induced changes in the number of MG may include both pre- and postsynaptic elements. This could be achieved by changes in neuronal processes but also via effects on neurogenesis or apoptosis, which should be explored in the future. Temperature may act either directly on neurons and/or glial cells or indirectly via effects on neurosecretory cells and/or the hormonal system (14, 15). Future studies at the ultrastructural level are needed to reveal the basis for changes in MG size.

In the lip of the MB calyx, changes in MG occurred at temperature differences ≤1°C. MG, therefore, represent a potential neuronal substrate for temperature-mediated effects on adult behavior (10). In the lip, numbers of MG were highest between 33.5 and 35°C, which overlaps with the narrow temperature range maintained in central brood cells (Fig. 4) (4). Pupae were kept at constant temperatures, whereas in the natural situation temperatures fluctuate in the range of ≈3°C, and fluctuations may be higher in peripheral brood cells (4). Therefore, effects in a natural population may be less extreme than they were in experimental animals. In the MB collar, MG numbers responded less sensitively, indicating that temperature has a differential influence on different brain neuropils. MG numbers were maintained after 1 week of sensory experience within the hive, indicating that bees were equipped with different numbers of synaptic units at the time when they performed "in-hive duties." A difference in the number of MG may affect subsequent maturation of the synaptic circuitry. Pupal-rearing temperature, therefore, may have important consequences for the ability of the adult MBs to express plastic changes in synaptic

structures and neuronal processes. The fact that temperature-mediated changes in the MBs are position- and modality-specific and do occur in a physiological range suggests a potential role in mediating behavioral plasticity, which may be especially true for complex tasks such as navigation, multimodal communication, learning, and memory (54, 55).

The division of labor in honey bee colonies is based on age polyethism and represents a most important feature of social life. Polyethism, however, was shown to be sensitive to environmental cues (2, 52). Different workers spend different amounts of time on nest activities compared with foraging, whereas others may never forage at all (53). We propose that pupal-rearing temperature may contribute to this flexibility of the division of labor and, therefore, may be an important component in the regulation of social life. Thermoregulation of brood rearing is also performed in other social insects. In ant colonies, this includes the selection of a suitable nest location (56) and thermoregulatory control of nest architecture (57, 58). Ant nurses cannot heat the brood actively but translocate preferentially pupae to adequate temperatures within the nest (59–61), indicating that, during pupal metamorphosis, the organism is most sensitive to temperature.

Phenotypic plasticity is the extent to which organisms change their development, physiology, morphology, and behavior in response to environmental cues. Social insects such as honey bees are excellent models for studies of mechanisms of environmentally caused effects on the nervous system and their consequences for behavior and social interactions. Future studies of temperature-mediated effects at the cellular and molecular levels are likely to provide important cues toward understanding the initial steps in neuronal and behavioral plasticity.

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