III Age-, task- and experience-dependent structural plasticity in *Apis mellifera* olfactory microglomerular complexes

Abstract

Worker honeybees proceed through a sequence of tasks, passing from hive and guard duties to foraging activities. However, the underlying neuronal changes mediating these behavioral transitions are not yet understood. Previous studies have shown that the mushroom bodies (MB), a brain region involved in sensory integration, learning and memory, undergo a volumetric expansion throughout adult life. Here we studied the mechanisms underlying MB structural changes by investigating age-, task-, and experience-dependent structural plasticity of microglomerular complexes in the lips of the MB, which exclusively process olfactory information. Presynaptic olfactory projection neuron boutons and postsynaptic dendritic spines of MB-intrinsic Kenyon cells forming microglomerular complexes (MC) were labeled simultaneously. A 3-D-based analysis of MC number, density and bouton size in the MB lips was performed in bees of different ages or the same age engaged in nursing and foraging behavior. Our results show that MB lip, MC number and density as well as bouton size stay constant over the first developmental stages, independent of environmental conditions. Further developmental and behavioral maturation leads to MB lip expansion accompanied by an increase in MC number and bouton size. Manipulations of social tasks cause a decrease in MC number compensated by an increase in bou-

ton size, while the MB lip hardly fluctuates. Thus continuity of developmental and behavioral maturation leads to structural plasticity expressed as an increase in MB lip volume, MC number and bouton size. Manipulations of age-related sensory perception induce degrading compensatory structural plasticity effects represented by a decrease in MC number and an increase in bouton size.

Introduction

Age- and experience-dependent neuronal changes are known to accompany naturally-occurring behavioral plasticity in vertebrates and invertebrates (Kolb and Whishaw, 1998; Fahrbach, 2006). Structural plasticity during experience-dependent maturation of the nervous system is common to many sensory systems (Wilson et al., 2006). Regarding olfactory systems, in rodents it has been shown that olfactory conditioning influences olfactory sensory neuron architecture (Kerr and Belluscio, 2006). Striking results are obtained for eusocial insects which provide the opportunity for a socio-neuroethological analysis of neuronal and behavioral maturation. The honeybee Apis mellifera has a discrete system of age-related division of labor (Roesch, 1925). Worker honeybees perform brood rearing ("nursing") for the first week of their adult life, engage in other hive maintenance duties when they are 2 - 3 weeks old, and subsequently carry out foraging (Winston, 1987). This schedule, however, is rather flexible, in order to meet the colony's varying needs, which are influenced by demographic and environmental conditions (Seeley, 2002). Such flexibility makes it possible to experimentally manipulate age- and experience-dependent changes in the bee brain, particularly in the mushroom bodies (MB) (Durst et al., 1994; Farris et al., 2001; Fahrbach, 2006).

The MBs are multisensory integration centers that play a dominat role in odor learning (Heisenberg, 1998). Their calyces receive second-order sensory input with different modalities and thus are believed to integrate sensory information,

possibly leading to context-dependent forms of learning (Menzel and Giurfa, 2001). Olfactory input is confined to the MB lip region by cholinergic excitatory projection neurons (PN) connecting the primary olfactory neuropil, the antennal lobe (AL) with the MB and transferring sensory information to the dendrites of the mushroom body-intrinsic neurons, the Kenyon cells (KC) (Mobbs, 1982; Sun et al., 1997; Abel et al., 2001). In the lip region of the MB calyces PN boutons and KC spines form microglomerular complexes (MC) (Frambach et al., 2004; Gronenberg, 2001). Each MC contains a PN presynaptic bouton as the central core, surrounded by a shell of KC dentritic tips (Frambach et al., 2004; Roessler et al., 2002; Yusuyama et al., 2002). It is known that the MBs integrate information from different neural circuits and undergo processes reflecting age- and experience-related plasticity.

Earlier studies by Withers et al. (1993) and Durst et al. (1994) showed that honeybee foragers exhibit a significantly larger MB volume than 1-day-old bees. Investigating neuronal architecture, Farris et al. (2001) showed that foraging experience correlates with the complexity of KC dendritic branching pattern and the number of KC dendritic spines. Our study aimed to search for the elemental structural components potentially underlying age-, task- and experience-dependent MB plasticity in the olfactory pathway. A combination of behavioral manipulations, pre- and postsynaptic labeling and 3-D-based quantification and volume measurement techniques was applied to investigate age- , task- and experience-dependent structural plasticity at the level of the MB lips. Our study reveals that MB structural plasticity depends on developmental and behavioral maturation as well age-adequate sensory perception.

Materials and Methods

Honeybees

Honeybees (*Apis mellicera carnica*) were obtained from colonies kept in our lab according to standard apicultural techniques. Experiments were performed during the summers of 2004 and 2005. Postnatal honeybees were obtained by removing brood combs containing late-stage pupae from a selected colony (headed by a naturally-mated queen) and transferring them – while protecting them from light – into an incubator (34 °C, 60-80 % relative humidity). The comb was inspected daily for newly-emerged bees which were collected and marked with different colored numbered tags (Opalithplättchen, Imkershop, Wolkenstein, Germany). The bees were then reintroduced into their original colonies or into a host colony reared within a net cage inside a glass house. Some newly-emerged bees were immediately used for histology (newborn bees, NB). To identify onset of orientation flights and later foraging behavior daily observations were made at the hive entrance for several sessions of 1 - 2 h each. Bees returning to the hive were designated foragers according to standard criteria (Lindauer, 1952; Winston, 1987). Pollen foragers showed conspicuous pollen loads or were dusted with pollen, whereas nectar foragers were identified by their swollen abdomens and checked for a nectar load by gently pressing the abdomen (nail probe). Observations of nursing behavior were made either by opening the hive or via an observation hive. Nurse (NU) bees were identified when they inserted their heads into brood cells (Farris et al., 2001). In addition, hypopharyngeal gland development was observed, which is known to be used for classification of NU and forager (FO) status (Winston, 1987; Farris et al., 2001).

Intracellular markings of a projection neurons

Electrodes (Hilgenberg) were pulled with a Brown-Flaming horizontal puller (P-2000 Sutter Instruments, Novarto, CA), and their tips were filled with 4 % tetramethylrhodamin-biotin dextran (Micro-Ruby; Molecular Probes, Eugene, OR) in 0.2 M potassium acetate. The electrodes were inserted into the AL, where recordings from PN were performed at about 50 - 180 μm depth. Electrode resistances in the tissue ranged from 140 to 200 MΩ. A silver wire placed into the eye served as the indifferent electrode. To ensure that the recording was performed from a PN Micro-Ruby was injected by using depolarizing pulses of 1 - 2 Hz and 0.2 second duration. Complete filling of the PN required dye injection for 30 - 45 minutes. After intracellular filling, the dye was allowed to diffuse for 3 hours.

Experimental part I: natural colonies under different environmental conditions

The colonies used for this experiment were either in a hive outside – referred to as the "rich" environment – or in a hive contained within a flight net situated in a glass house (the "poor" environment). In the rich environment the colony contained a naturally-mated queen, adult non-marked bees and about 700 newly-emerged marked bees. Foragers were marked with an additional paint mark to ensure a minimum of 2 days of foraging experience. The poor environment colony contained a single-inseminated active queen in a small hive with about 1000 adult bees and 300 newly-emerged marked bees. Foraging was performed at artificial grey odorless sugar feeders or by collecting pollen in a dark chamber. Behavioral observations were carried out as described above and bees of dedicated age (4, 14, 18, 37 days for the rich environment bees and 14, 18, 37 days for the poor environment) were collected.

Experimental part II: manipulations of social tasks in a single-cohort colony

The single-cohort colony of about 3000 bees used for this experiment was housed in an observation hive containing a single-inseminated queen. Observations of flight and foraging behavior were made as described above. Furthermore, observations of nursing and overall activity were made via the glass window of the observation hive. About 1000 bees were marked with numbered bee tags of different colors. Plastic discs were glued to the thoraxes of an additional 200 bees (following the procedure described by Fahrbach et al., 1998); these are referred to as "backpack" (BP) bees. The thickness of the plastic disk prevented BP bees from entering the comb to perform nursing behavior. Nevertheless, BP bees displayed good motor activity, trying to enter combs and interacting with other bees. The hive entrance was restricted such that only non-BP bees could enter or leave. Foragers were additionally marked with paint. At each adult developmental age (5, 10, 14, 18 days) BP, NU and FO bees were collected and dissected for histology.

Histology

Collected bees were immobilized by cooling. Brains were dissected in bee physiological saline solution (in $mmol^{-1}$: 136 NaCl, 3 KCl, 10 Na_2HPO_4, 2 KH_2PO_4, 105 sucrose, pH 6.7) and fixed in 4 % paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) (overnight at 4 °C). Brains were washed three times in PBS, embedded in 7 % low-melting agarose (Sigma) and sectioned in a frontal plane (100 μm) with a vibrating microtome (Leica, VT 1000 S). Agarose sections were transferred into marked titrimetric plates and preincubated in PBS with 0.2 % Triton-X100 (PBST) and 2 % normal goat serum (NGS; Sigma, St. Louis, MO) for 1 h at room temperature. For visualization of microglomerular complexes within the calycal lip we used a double labeling

technique adapted from Groh et al. (2004). This technique allows simultaneous pre- and postsynaptic labeling in the lip region of the MB calyx by combining synapsin with f-actin staining. Preparations were incubated simultaneously with a monoclonal antibody against the Drosophila synaptic-vesicle-associated protein synapsin I, (Klagges et al., 1996, 1:50; SynOrf1; kindly provided by E. Buchner, University of Wuerzburg, Wuerzburg, Germany) and with 0.2 units of Alexa Fluor 488 phalloidin (Molecular Probes, A-12379) in PBS for 2 days at 4 °C. To visualize synapsin, preparations were rinsed five times in PBS and incubated with a Cy5-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, PA; dilution 1:250 in PBS) overnight at 4 °C. For triple labeling of the intracellularly-stained PN, neuronal f-actin, and synapsin, the preparations containing marked PNs were fixed and processed as described above. To intensify the intracellular staining of the PN, streptavidin-Cy3 (Invitrogen, Kalsruhe, Germany), diluted 1:500, was added to the primary antiserum.

Confocal microscopy

Vibratome sections were imaged with a confocal laser scanning microscope (Leica TCS SP2) using a Leica HCX PL APO CS 40.0×1.25 OIL UV lens objective and a voxel size of $0.1 \times 0.1 \times 0.5\ \mu$m. For clear visualization of microglomerular complexes a defined zoom factor between 2 and 3 was used for each preparation. The preparation containing the intracellularly recorded and marked PN was imaged with a HC PL APO 20.0×0.70 IMM/CORR UV lens objective with a voxel size of $0.5 \times 0.5 \times 1\ \mu$m and a zoom factor of 1.4. Synapsin was visualized by the secondary antibody conjugated to Cy5 which was excited with the 633 nm line of a HeNe laser. F-actin stained with phalloidin was shown by a conjugated Alexa-488 which was excited using the 488 nm line of an ArKr laser. The PN staining was intensified by using streptavidin-Cy3 which was excited with the 543 nm line of a HeNe laser.

Quantification and volume estimation

To assure anatomical consistency lip volume, MC number, density as well as MC core volume represented by PN boutons was analyzed on optical sections at a defined plane in the central brain in the region of the central body. Lip volume and bouton volume as well as MC number and density was measured on images normalized to a voxel size of $0.15 \times 0.15 \times 1$,$\mu$m and a resolution of $796 \times 512 \times 11$. 3-D-based volumetric measurements and quantifications were performed using semiautomatic tools within the Amira 3.1.1 software (Mercury Computer Systems, Inc.). As a first step we applied a technique referred to as "segmentation" to assign voxel (volume pixel) as part of either the lip or a microglomerular complex to an appropriate label. The output provides a so-called "label field" image which consists of simple grey values which correspond to the object of interest. Lip and bouton volume as well as MC number and density were analyzed on a confocal stack (in total 11 μm thick) which was obtained using the same cropping method for all digitized confocal images. Each MC was identified by tracing (segmenting) the presynaptic PN boutons (stained with synapsin) in the x, y and z dimensions and aligning them with the second channel, which represented the postsynaptic KC spines (stained with phalloidin). Bouton tracing was performed blindly without any knowledge about the animals'group affiliation. Figure 1 illustrates the stepwise process of the 3-D-based volume measurement and quantification technique. Figure 1A shows the MC labeling, where the staining depicted in blue represents the presynaptic PN boutons labeled with synapsin and the one in red the postsynaptic KC spines labeled with phalloidin. The 3-D-based segmentation of the calycal lip region and the MC for the accordant x and y dimensions and the 11 μm thick z dimension is displayed in Figure 1B. The lip neuropil is outlined transparently, and the MC are depicted in blue. The MC label field (Fig. 1C) provides the basis for volumetric measurements as well as quantification which was accomplished by

using the "connected components" module of the Amira software. This module searches for connected regions in a 3-D image volume. The regions are detected based on thresholding, i.e., a region is a set of adjacent voxels with intensity values lying within a specific grey-value range (e.g. of the MC). Figure 1D depicts the screening 6-neighborhood algorithm used by the connected components module. A central voxel (green central square) is compared with its neighboring 6 voxels (surrounding green and red squares) in 3-D for the entire image stack and checked for connectivity. All connected voxels are assigned to one cluster and each cluster is counted, volumetric-measured and identified in the image's 3-D space.

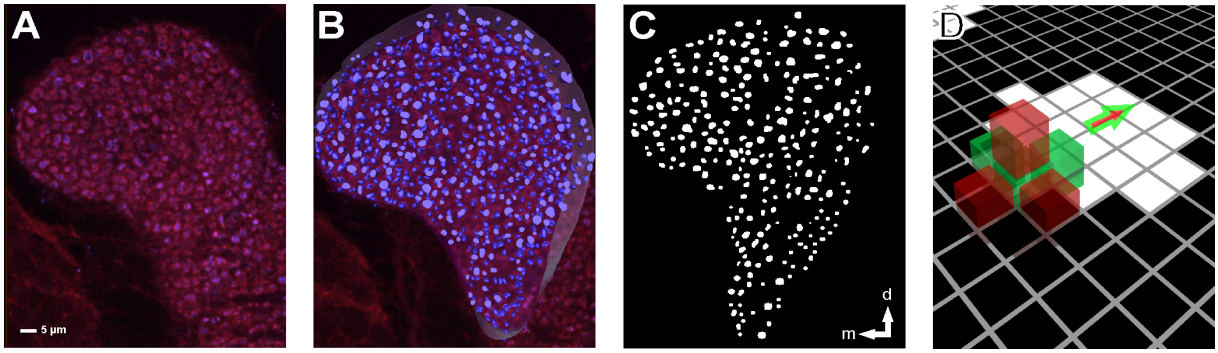

Figure III.1:
3-D volume measurement and quantification. An orthogonal view of a virtual slice showing the calycal lip. **A:** labelling of microglomerular complexes (MC) within the MB caycal lip. **B:** lip and MC segmentation, **C:** MC label field, **D:** 3-D volume measurement and quantification technique.

Data analysis

One-way ANOVA tests were performed to compare measured mean lip volume as well as mean MC number and mean bouton volume of all animals from the same group (statistical computation in R, `http://www.r-project.org`, Development Core Team, 2005). MC density was calculated by dividing the mean MC number over the accordant mean lip volume, both measured on an 11 μm thick z-stack. For statistical analysis of animals within one group a one-way ANOVA was used in combination with an all-pairwise multiple comparison

procedure, the Tukey Honest Significant Difference (Tukey HSD) test (Statistical Computation in R). Illustrations were created with Amira 3.1.1, MatLab, Adobe Illustrator CS2, Adobe Photoshop 7.0 and Corel Draw 11.

Results

Synaptic microglomerular complexes of projection neurons

The present study focussed on the analysis of structural changes at different levels of the MB lip region as related to the animals' age, behavior and experience. To prove the reliability and accessibility of the targeted structures, the MC complexes, using our staining methods, we first combined electrophysiological measurements of single PNs with immunohistochemistry. Figure 2 shows an intracellularly-marked single PN counterstained with the synapsin antibody SynOrf1 and the f-actin marker Alexa-488 phalloidin. The dendritic field of the single PN arborizing within the T3-56 glomerulus (square), identified according to Galizia et al. (1999) can be seen in Figure 2A. The PN leaves the AL via the median antennocerebral tract (mACT) and passes the MB posteriorly, sending collaterals via the inner ring tract to the lip region of the calyces and one collateral to the lateral horn of the protocerebrum. Its soma is located in the ventral rind of the AL (Fig. 2A, arrow). Figure 2B and C show neuronal branches (arrow outlined white) and axonal terminals with a large number of boutons (black arrows) which indicate presynaptic sites in the calycal lip. This triple labeling technique of a single PN with anti-synapsin and phalloidin employed here corroborates the results obtained by Frambach et al. (2004) and Groh et al. (2004) and shows that the antibody SynOrf1 marks presynaptic terminals of olfactory PN. Importantly, the phalloidin marker serves as a MC identifier representing a shell of KC dendritic tips surrounding the PN boutons as seen in electron microscopic sections (Ganeshina and Menzel, 2001; Ganeshina et al., 2006), allowing accurate quantification.

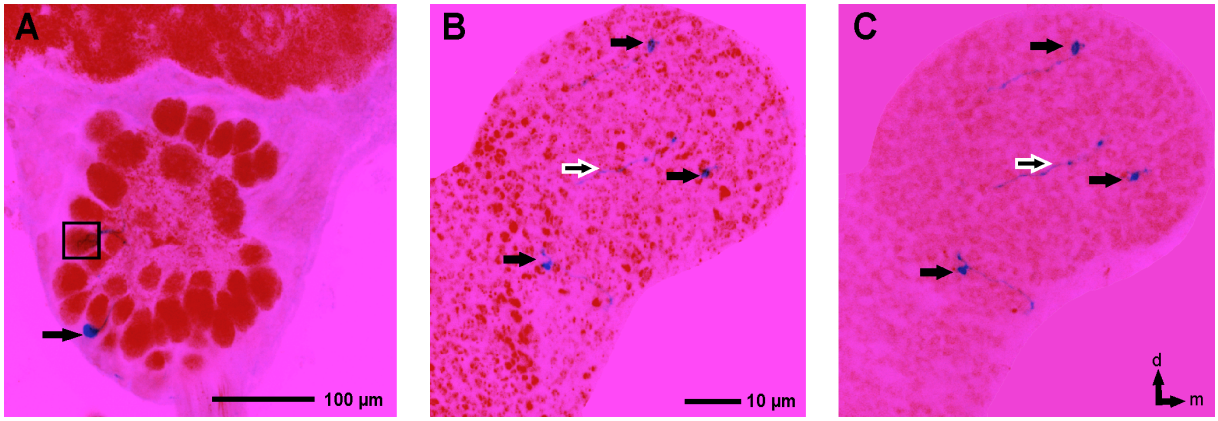

Figure III.2:
Microglomerular complexes of a single projection neuron (PN). An inverse shadow projection of a 3-D uniform scalar field. **A:** uniglomerular projections within the antennal lobe (AL). **B:** boutons of a single PN (blue) counterstained with a synapsin antibody (pink), **C:** boutons of a single PN (blue) counterstained with Alexa-488 phalloidin (pink).

Age-dependent structural plasticity and its dependence on environmental conditions

Previous studies have shown that MB calycal neuropils, in particular the lip and the collar region, undergo volumetric expansions during adult honeybee development and foraging experience (Withers et al., 1993; Durst et al., 1994; Fahrbach et al., 1998; Farris et al., 2001; Fahrbach et al., 2003). The goal of our study was to elucidate more precisely the age- and task-dependent forms of structural plasticity on the level of the MB and its microglomerular complexes (MC). We therefore focussed on the MB lips as an integration centre for sensory perception. Their volume, MC number and density as well as bouton volume were analyzed in bees at different developmental stages. The bees used (see Materials and Methods, Experimental part I) showed an onset of orientation flights between days 5 - 6. Pollen foraging was already observed in a few animals between day 9 - 11, with an increase around day 15. For the measured parameters, MB lip volume, MC number and density as well as bouton volume, we did not observe significant changes between nectar and pollen foragers, therefore they were pooled into one forager (Fo) group.

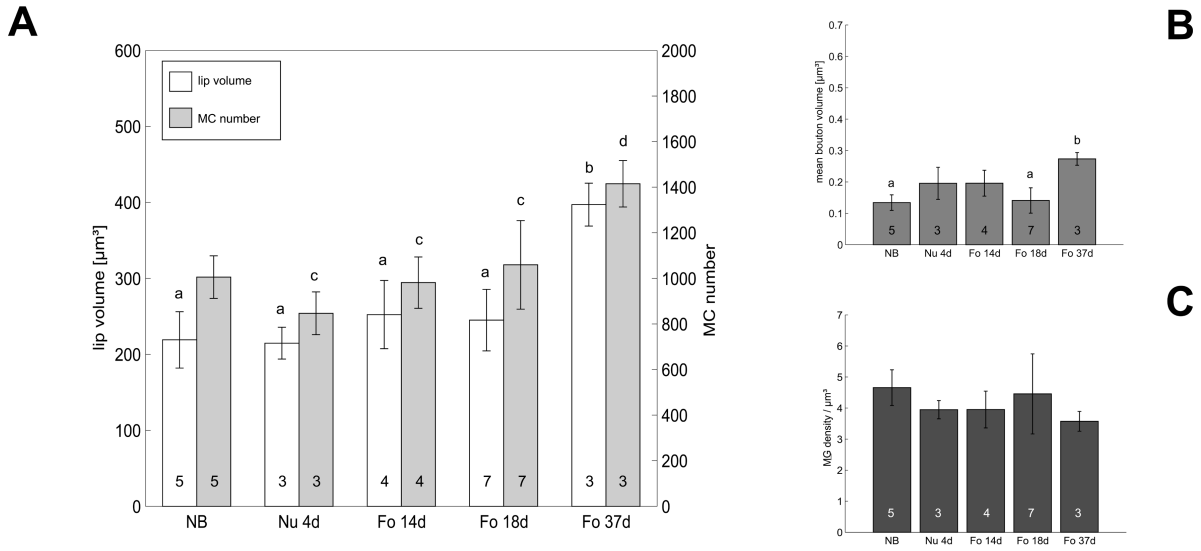

Figure III.3:
Age-dependent structural plasticity in bees reared in a "rich" environment (Fo). **A:** lip volume (one-way ANOVA (df = 4,15), F = 11.587; $p < 0.001$; Tukey HSD, $p < 0.001$) and MC number (one-way ANOVA (df = 4,15), F = 6.402; $p < 0.004$, Tukey HSD, $p < 0.002$). **B:** MC core (bouton) volume volume (one-way ANOVA (df = 4,15), F = 7.572; $p < 0.002$; Tukey HSD, $p < 0.002$). **D:** MC density (no significant differences). Sample size is depicted on each bar. Different letters indicate significant differences.

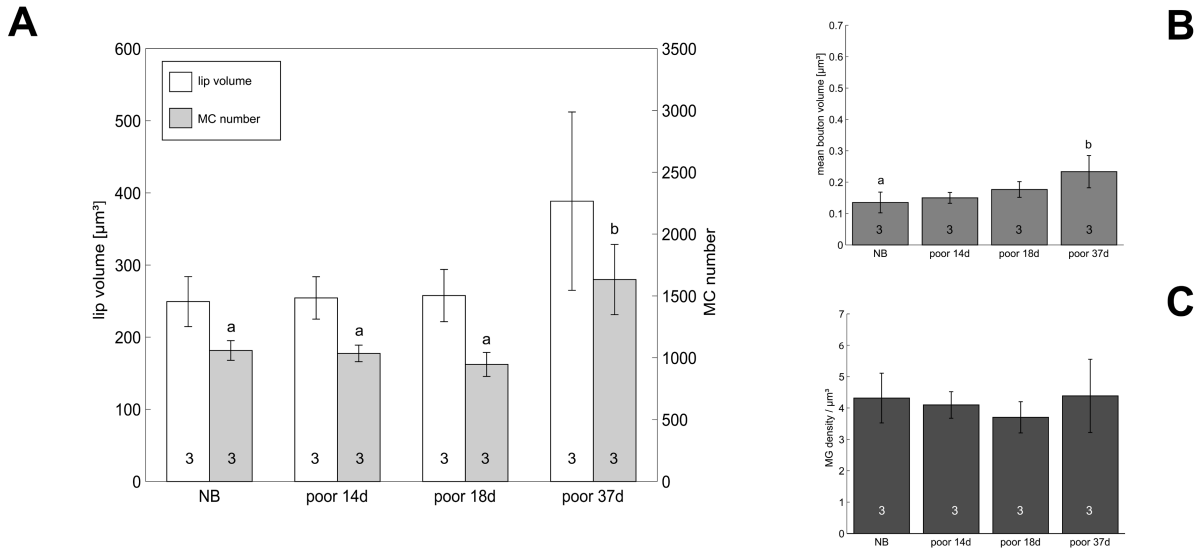

Figure III.4:
Age-dependent structural plasticity in bees reared in a "poor" environment(poor). **A:** lip volume (not significant differences but notable increase: $254 \pm 29\,\mu m^3$ on day 14 and $258 \pm 36\,\mu m^3$ on day 18 to $388 \pm 124\,\mu m^3$ on day 37), and MC number (one-way ANOVA (df = 3,8), F = 11.71; $p < 0.003$; Tukey HSD, $p < 0.004$). **B:** MC core (bouton) volume (one-way ANOVA (df = 3,8), F = 4.80; $p < 0.04$; Tukey HSD, $p < 0.04$. **D:** MC density (not significantly different).

Animals performing their age-related tasks in the natural environment ("rich" environment, Fig. 3) showed age-dependent structural plasticity during prolonged developmental maturation. Lip volume MC number and density as well as bouton volume stayed constant until the 18th day (Fig 3A - C). These three parameters increased significantly until the age of 37 days (Fig. 3A and B). As lip volume, MC number and bouton volume increased from day 18 to day 37, MC density did not change (Fig. 3C).

Similar age-dependent effects were observed in the group of animals reared under poor environmental conditions (Fig. 4). The colony with these animals was kept in a glass house and the animals collected sugar solution and pollen in a very small cage. Again lip volume, MC number, and bouton volume stayed constant until day 18 and then increased significantly in 37-day-old bees (Fig. 4A and B). MC density stayed constant throughout adult life, as it did in normally-behaving animals (Fig. 4C).

We did not observe statistical differences for the measured parameters comparing same-aged forager bees reared under either rich or poor environmental conditions. Thus environment-independent developmental and behavioral maturation until the age of day 18 does not lead to structural plasticity on the level of the MB lips, their number and density of MC as well as their bouton volume. The increase in lip volume, MC number and bouton volume is a late event in adult bee development, occurring between the 18^{th} and the 37^{th} days.

Manipulations of social tasks and sensory stimulations reveal age-dependent but not task-dependent plasticity

Manipulations of the bee's social life were used to demonstrate developmental maturation, task performance and experience and to determine the relationship between these factors and structural plasticity effects. These manipulations were achieved by rearing bees in a single-cohort hive in which same-aged bees

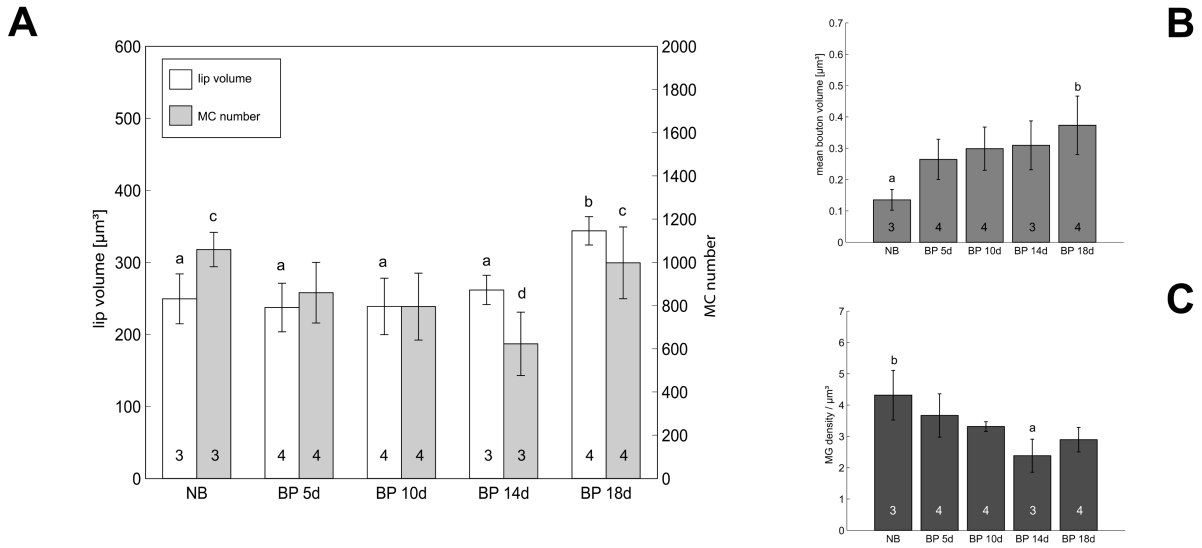

Figure III.5:
Age- and task-dependent structural plasticity in "backpack"(BP) bees prevented from nursing and foraging activities. **A:** increase in lip volume (one-way ANOVA (df = 4,13), F = 8.23 ; $p < 0.002$; Tukey HSD, $p < 0.003$) accompanied by a decrease in MC number which later returns to its initial value (one-way ANOVA (df = 4,13), F = 4.57; $p < 0.02$; Tukey HSD, $p < 0.02$. **B:** age-dependent increase of MC core (bouton) volume (one-way ANOVA (df = 4,13), F = 4.95; $p < 0.02$; Tukey HSD, $p < 0.007$). **C:** age-dependent decrease of MC density (one-way ANOVA (df = 4,13), F = 4.57, $p < 0.02$; Tukey HSD, $p < 0.005$. Sample size is depicted on each bar. Different letters indicate significant differences.

were prevented from nursing and foraging (backpacks, BP), or allowed to engage in nursing (Nu) and precocious foraging (pFO) (see Materials and Methods, Experimental part II). In these experiments bees could not be observed beyond the age of 18 days due to the instability of the single-cohort colony. Furthermore, nursing activities performed by the marked bees could be observed until day 14 presumably by virtue of newly-emerged bees engaged in nursing.

As observed in bees reared in a natural colony we found age-dependent structural plasticity effects, but these differed from those in a natural colony controlled by task performance and sensory stimulation. (Fig. 5, 6 and 7). In BP bees with the fewest social behaviors and receiving the fewest sensory stimulations, the lip volume stayed constant until the 14th day (Fig. 5A), accompanied by a decrease in MC number (Fig. 5A) and density (Fig. 5B) and an increase in bouton volume (Fig. 5C). On day 18 the two parameters lip volume, and MC

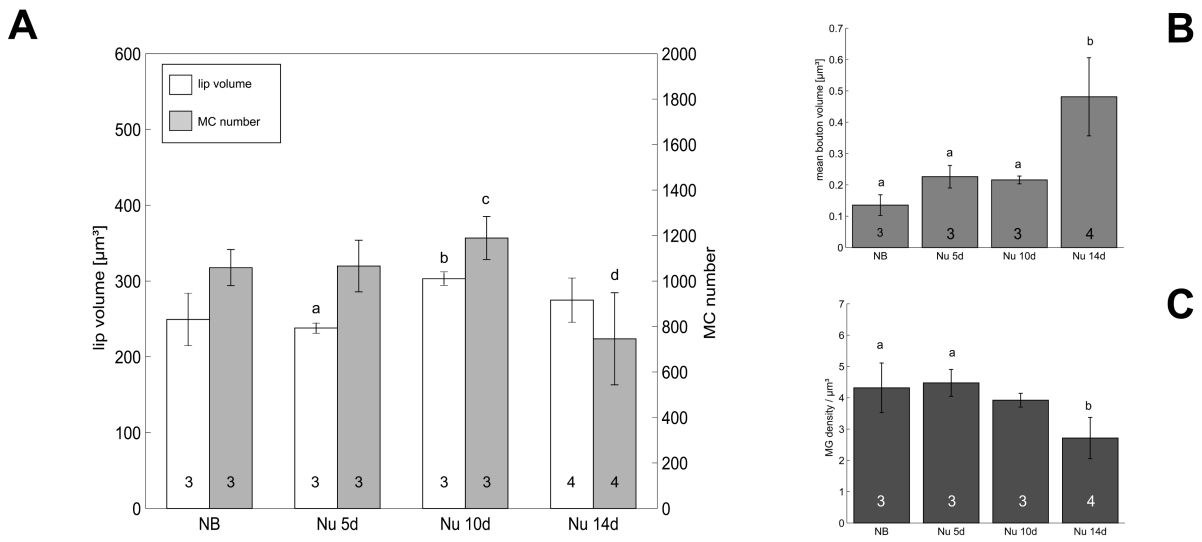

Figure III.6:
Age- and task-dependent structural plasticity observed in bees performing normal and extended nursing (Nu). **A:** task-specific increase in lip volume (one-way ANOVA (df = 3,9), F = 4.43; $p < 0.04$; Tukey HSD, p <ß,0.04) and MC number followed by a reduction (one-way ANOVA (df = 3,9) F = 6.51; $p < 0.02$; Tukey HSD, $p < 0.03$). **B:** age-dependent increase of MC core (bouton) volume on day 14 (one-way ANOVA (df = 3,9), F = 14.03; $p < 0.001$; Tukey HSD, $p < 0.003$) **C:** age-dependent decrease of MC density on day 14 (one-way ANOVA (df = 3,9), F = 6.75, $p < 0.02$; Tukey HSD, $p < 0.03$). Sample size is depicted on each bar. Different letters indicate significant differences.

number increased, and density stayed constant (Fig. 5A-C). Bees engaged in nursing behavior (Nu) showed almost no changes in lip volume (small increase on day 10, Fig. 6A), a decrease in MC number (small rise on day 10, Fig. 6A) and a bouton volume increase accompanied with a reduction of MC density until the 14^{th} day (Fig. 6B,C). In pFO no change of lip volume was seen over the whole lifespan (18^{th} day, Fig. 7A). The number of MC showed a tendency to decrease, while bouton volume increased until day 14 (Fig. 7B). The increase of MC volume was associated with a decrease in MC density (Fig. 7C).

The most striking discrepancy between BP, Nu and pFo bees was observed in the temporal dynamics of the structural plasticity effects. The MC number and density decreased in BP bees from day 5 on (Fig. 5A and C) whereas in Nu bees this did not occur until day 14 (Fig. 6A and C). In pFo bees we observed a correlation between the decrease in MC number and density from day 5 on-

wards. The bouton volume increase is accompanied with MC density decrease in all three groups (Fig. 5B, Fig. 6B, Fig. 7B). Collective consideration of

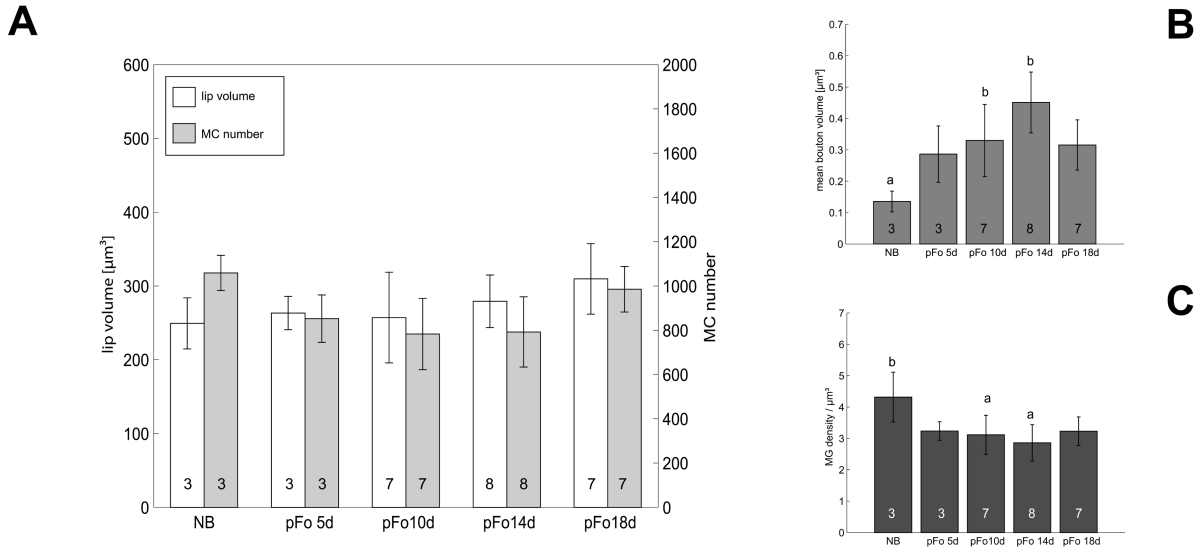

Figure III.7:
Age- and task-dependent structural plasticity observed in precocious foragers (pFO). **A:** Lip volume and MC number during adult development (no significant differences). **B:** MC core (bouton) volume increase (one-way ANOVA (df = 4,23) F = 6.75; $p < 0.001$; Tukey HSD, $p < 0.001$). **C:** MC density decrease (one-way ANOVA (df = 4,23), F = 3.75, $p < 0.03$, Tukey HSD, $p < 0.01$). Sample size is depicted on each bar. Different letters indicate significant differences.

the three groups (BP, Nu, pFo) in which social tasks and thus the age-related sensory stimulations and behaviors were disturbed indicates that MB plasticity is expressed in terms of a reduction of MC number and an increase in bouton volume against a background of no change in lip volume, leading to a reduction in MC density. Figure 8 illustrates this effect more precisely by comparing lip volume, MC number and density as well as bouton volume in 14-day-old Fo (natural colony under rich conditions), poor (natural colony under poor conditions), BP (single-cohort, prevented from nursing and foraging), Nu (nursing) and pFo (precocious foraging) bees. Lip volume stays constant for all groups (Fig. 8A). MC number is significantly lower only in BP bees, but also shows a tendency toward reduction in Nu and pFO bees. MC density decreased significantly in all three manipulated groups (Fig. 8A and C); bouton volume is

significantly higher in Nu and pFO bees and shows a tendency to increase in BP bees (Fig. 8B). The most conspicuous decrease in MC number was observed in 14-day-old BP bees (Fig. 8A) indicating that the most reduced social behavior and the least sensory stimulations leads to the largest effects.

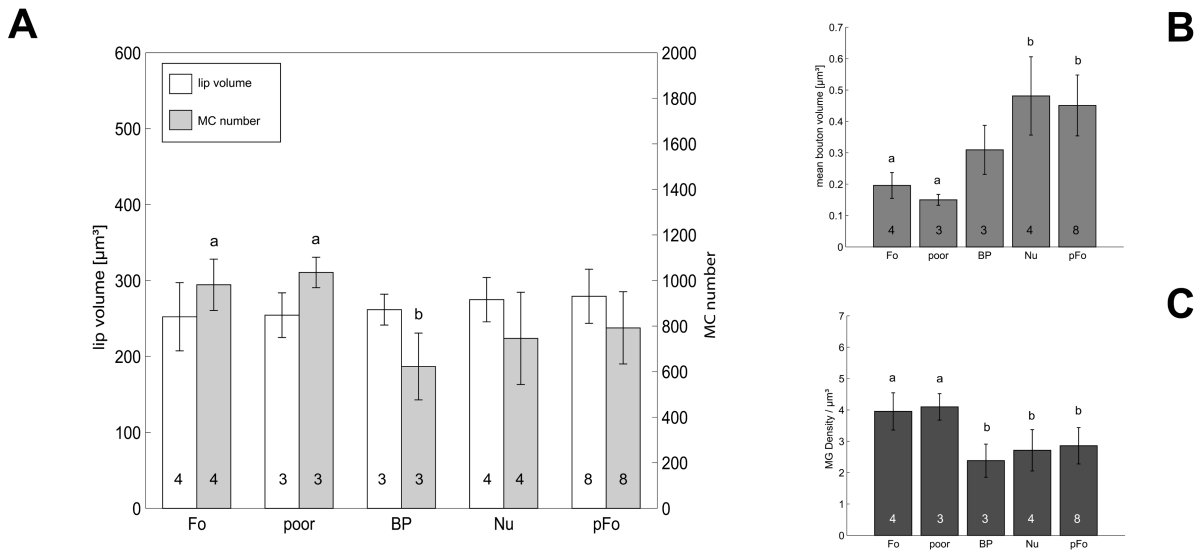

Figure III.8:
Comparison between 14-day-old foragers from a natural colony reared under rich conditions (Fo), foragers from a natural colony reared under poor conditions (caged in a glass house), single-cohort bees prevented from nursing and foraging (BP), single-cohort nurses and extended nurses (Nu) and single-cohort precocious foragers (pFo). **A:** no differences in lip volume but a decreased MC number. (Comparison of single-cohort bees with natural colony bees: one-way ANOVA ($df = 4,21$) $F = 4.22$; $p < 0.02$; Tukey HSD, $p < 0.05$). **B:** MC core (bouton) volume (comparison of single-cohort bees with natural colony bees: one-way ANOVA ($df = 4,21$) $F = 12.13$; $p < 0.001$; Tukey HSD, $p < 0.003$). **C:** MC density (comparison of single cohort bees to natural colony bees: one-way ANOVA ($df = 4,21$) $F = 6.63$; $p < 0.003$; Tukey HSD, $p < 0.05$).

Discussion

This study provides the first 3-D-based analysis of pre- and postsynaptic elements within a defined microcircuit of an invertebrate. We visualized microglomerular complexes (MC) within the mushroom body (MB) lips of the honeybee by simultaneously labelling pre- and postsynaptic components (Fig. 1). A 3-D-based quantification and volumetric measurement technique allowed us to trace MB structural changes underlying elemental components as far as

possible with light microscopical techniques. We also aimed for a better distinction between effects of environmental experience and those that result from the bees'social life.

In contrast to previous studies (Withers et al., 1993; Durst et al., 1994; Fahrbach et al., 1998; Farris et al., 2001) our results show that the normal succession of behavioral tasks under natural environmental conditions is not accompanied by volume changes in the lip region of the MB, and that the increase in volume is a late event in adult bee development occurring between the 18th and the 37th days of age. Neither manipulations of the environmental conditions nor of social tasks lead to volume changes in the lip during the first phase of adult life. Our data do not support the hypothesis proposed by Farris et al. (2001) that lip volume expansion in the mushroom bodies is an ongoing process during honeybee behavioral development, initiated before the onset of foraging. We also cannot support the conclusion reached by Ismail et al. (2005), who predicted that foraging experience is one of the major components controlling MB plasticity since precocious foragers did not have a larger lip volume than same-aged bees inside the hive. The reasons for these discrepancies might be related to the histological procedures applied in the present and former studies, possibly indicating that the shrinking effects caused by histological processes affected the age- and experience-related stages differently. Another possibility relates to the different ways volume measures were collected. We reconstructed the complete volumes of the lip in all animals using optical sections from the confocal microscope. The other studies calculated volumes from selected sections. These stereological methods interpreted 3-D structures on the basis of 2-D sections. We believe that our 3-D techniques are less prone to failures, but as long as in-vivo measurements are not performed we cannot rule out that our histological procedures also produce artifacts. Since all brains in our study were treated exactly the same way, we can at least exclude the possibility that the differences between the groups are caused by different procedures.

Nectar-and pollen-forager's behavior differs in many respects (Page et al., 2006) and thus we analyzed them separately. Since we did not find significant differences in the volume measures, the number of MC and their density between nectar and pollen foragers on day 18, we exclude the possibility of different forms of experience by these two foraging tasks and pooled the data. This finding is consistent with the results observed by Withers et al. (2006), who reported on changes in the volume of the mushroom bodies which are temporally associated with the performance of foraging but do not depend of foraging experience.

Manipulations of the bees'social life as well as their age-and task-related sensory stimulations revealed drastic effects on the MC number, density and bouton volume, which can be disconnected from age effects because we compared same-aged bees housed in the same colony. In such a "cohort colony" bees perform different tasks even though they are of the same age, leading to both advancement and slowdown of the age-related processes controlling behavior. We manipulated bees such that they either could not engage in certain tasks like feeding the larvae, cleaning cells and leaving the hive (BP bees), or so that they performed prolonged nursing (Nu) and precocious foraging (pFo) behavior. All bees in the cohort colony showed the same structural plasticity effects in terms of a constant lip volume associated with a reduction in MC number and density compensated by an increase in bouton volume (Fig. 8A - C). Thus the causes for these common structural effects cannot be found in the various tasks that these bees performed or the different sensory stimulations that they received. Rather, it must be related to coordination between age and sensory stimulation and / or performances. Indeed, developmental programs are known to be tuned to the naturally-occurring sensory stimulation and / or behavioral performances.

Studies on the zebra finch *Taeniopygia guttata* have shown that an extension of the sensitive period in sexual imprinting can be postponed to later ages by sensory deprivation or inadequate stimulation, causing spine density changes

(Bischof et al., 2002).

For the honeybee – as a social animal – it is known that behavioral adaptations correlate with changes in the hormonal control mainly the titer of juvenile hormone (Robinson, 2006); this means that the age-related programs expressed in the respective hormonal controls appear to prepare the animal for particular sensory / motor conditions and thus coordinate the formation of the neural structures to the expected inputs and / or outputs. Since the general effect of disturbing such coordination was the reduction of MC number accompanied by an increase in the bouton volumes against the background of a constant lip volume, it is tempting to conclude that the MC are the elementary modules where such coordination is structurally expressed. This interpretation is supported by the finding that the temporal dynamics of the observed structural plasticity effect differ between the three groups (see the time courses for BP, Nu and pFO in Figs. 5, 6 and 7, respectively). The most distinct decrease in MC number and density and increase in bouton volume occurred at the earliest developmental stage (day 5) in bees with fewest task-performances and sensory stimulations (Fig. 5), and the related changes occurred later in the other two groups.

Our results do not provide us with the opportunity to identify the primary factor, decrease of MC number, or increase in bouton volume. Since MC number and bouton volume increase later in adult life under natural conditions in which sensory stimulation and behavioral performances increased, one might argue that the number of MC is the critical factor reacting sensitively to the coordination of age-related developmental programs and experience, and the fact that the bouton volume is the secondary parameter against the background of a constant volume of the lip region. The observed changes in MC quantity might induce a remodeling process (Stepanyants et al., 2002) at their postsynaptic sites, the formation or elimination of KC dentritic spines (postsynaptic sites) or changes in the arborization pattern of KCs and inhibitory feedback neurons from the MB lobes, both of which may serve as presynaptic sites to the PN bouton.

This would lead to the reorganization of the microcircuit around each bouton (Ganeshina and Menzel, 2001) and change its organization not only quantitatively with respect to synaptic weights but also qualitatively by rewiring the connectivity within each MC. At the presynaptic level of the MC the increase in bouton volume suggests that the PN increase their presynaptic vesicle content, the number of postsynaptic targets and thus the efficacy of their synaptic output, a potential process for compensating a reduction in MC number.

The effect of neuronal pruning and / or changes in synaptic efficacy is most pronounced in BP bees that were prevented from both nursing and foraging (Fig. 5). Studies in mice showed that depriving an eye of visual stimulation leads to an activity-dependent decrease in the effectiveness of deprived synapses in the visual cortex (Bear, 1995). Naegerl et al. (2004) found that spines on CA1 pyramidal neurons undergo bidirectional activity-dependent morphological plasticity. This phenomenon might explain the combined effect of decrease in MC number and increase in bouton volume if one considers the regulation of MC number as a presynaptic effect and the increase in bouton volume as a postsynaptic effect. Further studies need to elucidate the relationship between physiological and anatomical plasticity as related to this compensatory plasticity in the MB of the bee. Physiological and structural studies need to be brought to the single neuron level, taking advantage of the insect nervous system with its identified neurons and well-described neural nets (Abel et al., 2001; Gronenberg, 2001; Kirschner et al., 2006). Intracellularly marked neurons can be reconstructed in-vivo using the two-photon microscope (Wachowiak et al., 2004; Franke and Menzel, unpublished observations). The strength of synaptic transmission can be tested in Ca-imaging experiments (Szyszka et al., 2005), and electronmicroscopy studies can be combined with single-cell marking and immunolabeling (Ganeshina and Menzel, unpublished). Artefacts induced by histological procedures need to be avoided in the future, and complete 3-D reconstructions of in-vivo structures are possible for the whole bee brain using the

two-photon microscope; these will become the standard in the field. This will allow us to test the hypothesis posed here which states that the change in MC number is the primary factor in plasticity induced by uncoupling age-dependent developmental programs with related sensory / motor performances. It will be necessary to test whether the efficiency of the synaptic connection and its wiring are adapted to these changes.