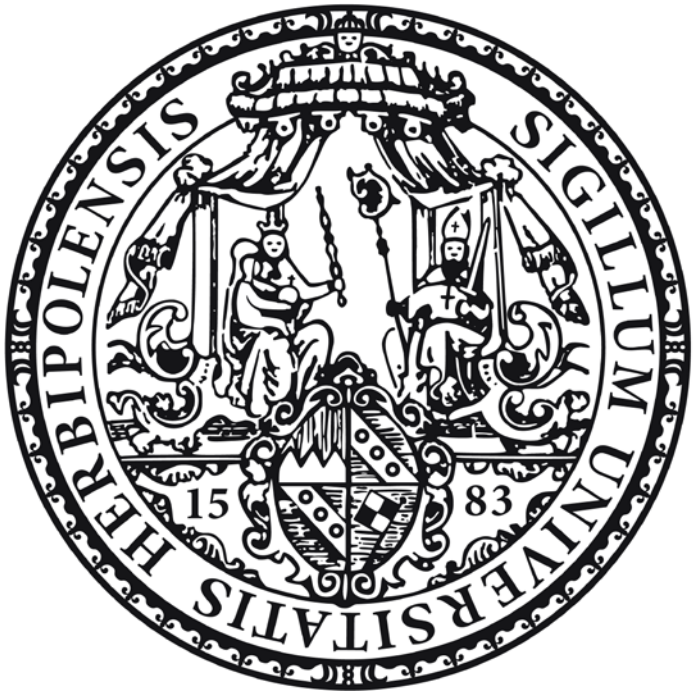
Aspects of neuronal plasticity in the mushroom body calyx during adult maturation in the honeybee *Apis mellifera*



Dissertation zur Erlangung des

Naturwissenschaftlichen Doktorgrades

der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

Thomas Sebastian Münz

aus Rieden am Forggensee

Würzburg 2015

Eingereicht am: ……

Mitglieder der Promotionskommission:

Vorsitzender: Prof. Dr. Markus Engstler (Universität Würzburg)

Gutachter: Prof. Dr. Wolfgang Rössler (Universität Würzburg)

Gutachter: Prof. Dr. Alison Mercer (University of Otago, New Zealand)

Tag des Promotionskolloquiums: ……..

Doktorurkunde ausgehändigt am: …….

Affidavit

(Eidesstattliche Erklärung)

gemäß §4 Abs. 3 Ziff. 3, 5 and 8

der „Promotionsordnung der Julius-Maximilians-Universität Würzburg“

Hiermit erkläre ich an Eides statt, das die Dissertation “Aspects of neuronal plasticity in the mushroom body calyx during adult maturation in the honeybee *Apis mellifera*” selbständig, unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt wurde.

Weiterhin habe ich noch keinen Promotionsversuch unternommen, oder diese Dissertation in gleicher oder ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt.

Würzburg, 25.01.2015 _____

Unterschrift

This thesis is based on the following manuscripts:

- Muenz TS, Groh C, Maisonnasse A, Le Conte Y, Plettner E, Rössler W. 2015. Neuronal plasticity in the mushroom body calyx during adult maturation in the honeybee and possible pheromonal influences. **under revision**
- Pasch E*, Muenz TS*, Rössler W. 2011. CaMKII is differentially localized in synaptic regions of Kenyon cells within the mushroom bodies of the honeybee brain. **Journal of Comparative Neurology** 519:3700–3712.
  *equally contributing first authors
- Muenz TS, Maisonnasse A, Le Conte Y, Plettner E, Rössler W. 2012. Sensory reception of the primer pheromone ethyl oleate. **Naturwissenschaften** 99:421-425

"Dissertation Based on Several Published Manuscripts"

Statement on individual author contributions and on legal second publication rights

Publication (complete reference): Muenz TS, Groh C, Maisonnasse A, Le Conte Y, Plettner E, Rössler W. 2015. Neuronal plasticity in the mushroom-body calyx during adult maturation in the honeybee and possible pheromonal influences. **under revision**					
Participated in	**Author-Initials,** Responsibility decreasing from left to right				
Study Design	TSM	WR	CG	YLC, EP	AM
Data Collection	TSM, CG	AM			
Data-Analysis and Interpretation	TSM, CG	AM	WR		
Manuscript Writing	TSM	WR	CG		

Publication (complete reference): Pasch E*, Muenz TS*, Rössler W. 2011. CaMKII is differentially localized in synaptic regions of Kenyon cells within the mushroom bodies of the honeybee brain**. Journal of Comparative Neurology** 519:3700–3712. *equally contributing first authors					
Participated in	**Author-Initials,** Responsibility decreasing from left to right				
Study Design	TSM	WR	PE		
Data Collection	PE	TSM			
Data-Analysis and Interpretation	TSM	PE	WR		
Manuscript Writing	PE, TSM	WR			

Publication (complete reference): Muenz TS, Maisonnasse A, Le Conte Y, Plettner E, Rössler W. 2012. Sensory reception of the primer pheromone ethyl oleate. **Naturwissenschaften** 99:421-425					
Participated in	**Author-Initials,** Responsibility decreasing from left to right				
Study Design	TSM	AM	WR	YLC, EP	
Data Collection	TSM, AM				
Data-Analysis and Interpretation	TSM, AM	WR	YLC	EP	
Manuscript Writing	TSM	WR	AM	YLC, EP	

I confirm that I have obtained permission from both the publishers and the co-authors for legal second publication.

I also confirm my primary supervisor's acceptance.

_____

Doctoral Researcher's Name Date Place Signature

It is not the mountain we conquer, but ourselves.

- Sir Edmund Hillary (*1919 - +2008)

Table of contents

Summary

Division of labor represents a major advantage of social insect communities that accounts for their enormous ecological success. In colonies of the honeybee, *Apis mellifera*, division of labor comprises different tasks of fertile queens and drones (males) and, in general, sterile female workers. Division of labor also occurs among workers in form of an age-related polyethism. This helps them to deal with the great variety of tasks within the colony. After adult eclosion, workers spend around three weeks with various duties inside the hive such as tending the brood or cleaning and building cells. After this period workers switch to outdoor tasks and become foragers collecting nectar, pollen and water. With this behavioral transition, workers face tremendous changes in their sensory environment. In particular, visual sensory stimuli become important, but also the olfactory world changes. Foragers have to perform a completely new behavioral repertoire ranging from long distance navigation based on landmark orientation and polarized-skylight information to learning and memory tasks associated with finding profitable food sources. However, behavioral maturation is not a purely age-related internal program associated with a change, for example, in juvenile hormone titers. External factors such as primer pheromones like the brood pheromone or queen mandibular pheromone can modulate the timing of this transition. In this way colonies are able to flexibly adjust their work force distribution between indoor and outdoor tasks depending on the actual needs of the colony. Besides certain physiological changes, mainly affecting glandular tissue, the transition from indoor to outdoor tasks requires significant adaptations in sensory and higher-order integration centers of the brain.

The mushroom bodies integrate olfactory, visual, gustatory and mechanosensory information. Furthermore, they play important roles in learning and memory processes. It is therefore not surprising that the mushroom bodies, in particular their main input region, the calyx, undergo volumetric neuronal plasticity. Similar to behavioral maturation, plastic changes of the mushroom bodies are associated with age, but are also to be affected by modulating factors such as task and experience.

In my thesis, I analyzed in detail the neuronal processes underlying volumetric plasticity in the mushroom body. Immunohistochemical labeling of synaptic proteins combined with

quantitative 3D confocal imaging revealed that the volume increase of the mushroom body calyx is largely caused by the growth of the Kenyon cell dendritic network. This outgrowth is accompanied by changes in the synaptic architecture of the mushroom body calyx, which is organized in a distinct pattern of synaptic complexes, so called microglomeruli. During the first week of natural adult maturation microglomeruli remain constant in total number. With subsequent behavioral transition from indoor duties to foraging, microglomeruli are pruned while the Kenyon cell dendritic network is still growing. As a result of these processes, the mushroom body calyx neuropil volume enlarges while the total number of microgloumeruli becomes reduced in foragers compared to indoor workers. In the visual subcompartments (calyx collar) this process is induced by visual sensory stimuli as the beginning of pruning correlates with the time window when workers start their first orientation flights. The high level of analysis of cellular and subcellular process underlying structural plasticity of the mushroom body calyx during natural maturation will serve as a framework for future investigations of behavioral plasticity in the honeybee.

The transition to foraging is not purely age-dependent, but gets modulated, for example, by the presence of foragers. Ethyl oleate, a primer pheromone that is present only in foragers, was shown to delay the onset of foraging in nurse bees. Using artificial application of additional ethyl oleate in triple cohort colonies, I tested whether it directly affects adult neuronal plasticity in the visual input region of the mushroom body calyx. As the pheromonal treatment failed to induce a clear behavioral phenotype (delayed onset of foraging) it was not possible to show a direct link between the exposure to additional ethyl oleate and neuronal plasticity in mushroom body calyx. However, the general results on synaptic maturation confirmed my data of natural maturation processes in the mushroom body calyx.

Given the result that dendritic plasticity is a major contributor to neuronal plasticity in the mushroom body calyx associated with division of labor, the question arose which proteins could be involved in mediating these effects. Calcium/calmodulin-dependent protein kinase II (CaMKII) especially in mammals, but also in insects (*Drosophila*, Cockroach), was shown to be involved in facilitating learning and memory processes like long-term synaptic potentiation. In addition to presynaptic effects, the protein was also revealed to directly interact with cytoskeleton elements in the postsynapse. It therefore is a likely candidate to mediate structural synaptic plasticity. As part of my thesis, the presence and distribution of

CaMKII was analyzed, and the results showed that the protein is highly concentrated in a distinct subpopulation of the mushroom body intrinsic neurons, the noncompact Kenyon cells. The dendritic network of this population arborizes in two calyx subregions: one receiving mainly olfactory input – the lip – and the collar receiving visual input. This distribution pattern did not change with age or task. The high concentration of CaMKII in dendritic spines and its overlap with f-actin indicates that CaMKII could be a key player inducing structural neuronal plasticity associated with learning and memory formation and/or behavioral transitions related to division of labor. Interestingly CaMKII immunoreactivity was absent in the basal ring, another subregion of the mushroom body calyx formed almost exclusively by the inner compact Kenyon cells and known to receive combined visual and olfactory input. This indicates differences of this mushroom body subregion regarding the molecular mechanisms controlling plastic changes in corresponding Kenyon cells.

How is timing of behavioral and neuronal plasticity regulated? The primer pheromone ethyl oleate was found in high concentrations on foragers and was shown to influence behavioral maturation by delaying the onset of foraging when artificially applied in elevated concentrations. But how is ethyl oleate transferred and how does it shift the work force distribution between indoor and outdoor tasks? Previous work showed that ethyl oleate concentrations are highest in the honeycrop of foragers and suggested that it is transferred and communicated inside the colony via trophallaxis. The results of this thesis however clearly show, that ethyl oleate was not present inside the honey crop or the regurgitate, but rather in the surrounding tissue of the honey crop. As additionally the second highest concentration of ethyl oleate was measured on the surface of the cuticle of forgers, trophallaxis was ruled out as a mode of transmission. Neurophysiological measurements at the level of the antennae (electroantennogram recordings) and the first olfactory neuropil (calcium imaging of activity in the antennal lobe) revealed that the primer pheromone ethyl oleate is received and processed as an olfactory stimulus. Appetitive olfactory conditioning using the proboscis extension response as a behavioral paradigm showed that ethyl oleate can be associated with a sugar reward. This indicates that workers are able to perceive, learn and memorize the presence of this pheromone. As ethyl oleate had to be presented by a heated stimulation device at close range, it can be concluded that this primer pheromone

acts via close range/contact chemoreception through the olfactory system. This is also supported by previous behavioral observations.

Taken together, the findings presented in this thesis revealed structural changes in the synaptic architecture of the mushroom body calyx associated with division of labor. For the primer pheromone ethyl oleate, which modulates the transition from nursing to foraging, the results clearly showed that it is received via the olfactory system and presumably acts via this pathway. However, manipulation experiments did not indicate a direct effect of ethyl oleate on synaptic plasticity. At the molecular level, CaMKII is a prime candidate to mediate structural synaptic plasticity in the mushroom body calyx. Future combined structural and functional experiments are needed to finally link the activity of primer pheromones like ethyl oleate to the molecular pathways mediating behavioral and synaptic plasticity associated with division of labor in *Apis mellifera*. The here identified underlying processes will serve as excellent models for a general understanding of fundamental mechanisms promoting behavioral plasticity.

Zusammenfassung

Arbeitsteilung stellt einen der wesentlichen Faktoren dar, der für den ökologischen Erfolg von sozialen Insektengemeinschaften verantwortlich ist. In Staaten der Honigbiene, *Apis mellifera*, umfasst die Arbeitsteilung verschiedene Aufgaben für die fertilen Königinnen und Drohnen (Männchen) beziehungsweise die gewöhnlicherweise sterilen Arbeiterinnen. Arbeitsteilung findet aber auch in Form eines altersabhängigen Polyethismus zwischen den Arbeiterinnen selber statt. Dies hilft ihnen die Vielzahl verschiedener Aufgaben im Stock zu bewältigen. Nach dem Schlupf verbringen die Arbeiterinnen etwa drei Wochen mit verschiedenen Aufgaben im Stock, wie beispielsweise Brutpflege oder Reinigen und Bauen neuer Wabenzellen. Nach dieser Zeit wechseln die Arbeiterinnen zu Aufgaben außerhalb des Stocks und werden Nektar-, Pollen- oder Wassersammlerinnen. Durch diesen Verhaltensübergang sind die Arbeiterinnen mit einem massiven Wandel ihrer sensorischen Umwelt konfrontiert. Im speziellen werden nun visuelle Reize wichtig, aber auch die olfaktorische Welt der Arbeiterinnen ändert sich. Sammlerinnen zeigen ein komplett neues Verhaltensrepertoire das von Langstreckennavigation, basierend Landmarken und dem Polarisationsmuster des Himmels, bishin zu Lern- und Gedächtnisaufgaben im Zusammenhang mit dem Auffinden profitabler Futterquellen reicht. Allerdings ist Verhaltensreifung kein rein altersbedingtes internes Programm beispielsweise basierend auf einer Veränderung des Juvenilhormon-Titers. Externe Faktoren wie beispielsweise die Primer Pheromone Brutpheromone oder Königinnenpheromon können den Zeitpunkt des Übergangs modulieren. Hierdurch sind Staaten in der Lage ihre Arbeiterkräfte flexibel zwischen Innen- und Außendienst Aufgaben zu verschieben. Neben bestimmten physiologischen Veränderungen, die vor allem Drüsengewebe betreffen, benötigt der Übergang vom Innendienst zum Außendienst deutliche Anpassungen sensorischer und höherer Integrationszentren im Gehirn.

Die Pilzkörper integrieren olfaktorische, visuelle und mechanosensorische Informationen. Sie spielen weiterhin eine wichtige Rolle für Lern- und Gedächtnisvorgänge. Es ist daher nicht überraschend, dass die Pilzkörper, im Speziellen deren Haupteingangsregion, der Kalyx, eine neuronale Volumensplastizität durchlaufen. Ähnlich wie die Verhaltensreifung, sind plastische Veränderungen im Pilzkörper mit dem Alter verbunden, werden aber auch durch modulierende Faktoren wie Aufgabe und Erfahrungen beeinflusst.

In meiner Dissertation habe ich detailliert die neuronalen Prozesse analysiert, die der Volumensplastizität des Pilzkörpers zugrunde liegen. Immunhistologische Färbungen synaptischer Proteine kombiniert mit quantitativer 3D Konfokalmikroskopie zeigten, dass die Volumenszunahme des Pilzkörpers hauptsächlich durch dendritisches Wachstum des Kenyon-Zellen-Netzwerks bedingt ist. Dieses Auswachsen wurde begleitet durch Veränderungen der synaptischen Architektur des Kalyx des Pilzkörpers, welcher in Form synaptischer Komplexe, sogenannter Mikroglomeruli organisiert ist. Während der ersten Woche der Adultreifung blieb die Gesamtzahl der Mikroglomeruli konstant. Im folgenden Verhaltensübergang von Innendienstaufgaben zum Sammeln, wurden die Mikroglomeruli zurückgetrimmt, während das dendritische Kenyon-Zell-Netzwerk weiterhin wuchs. Als Ergebnis dieser Prozesse vergrößerte sich das Volumen des Kalyx des Pilzkörpers während die Gesamtzahl der Mikroglomeruli bei Sammlerinnen im Vergleich zu Inndienst Arbeiterinnen reduziert war. In der visuellen Unterregion (Kragen des Kalyx) wurde dieser Prozess induziert durch sensorische Stimuli, da der Beginn des Zurücktrimmens mit dem Zeitfenster zusammenfiel, in dem die Arbeiterinnen ihre ersten Orientierungsflüge starteten. Der hohe Analysegrad der zellulären und subzellulären Prozesse, die der strukturellen Plastizität des Kalyx des Pilzkörpers während der natürlichen Reifung zugrunde liegen, wird zukünftigen Untersuchungen der Verhaltensplastizität bei Honigbienen als Referenz dienen.

Der Übergang zur Sammlerin ist nicht rein altersabhängig, sondern wird beispielsweise durch die Gegenwart von anderen Sammlerinnen moduliert. Ethyloleat, ein Primer Pheromone das nur auf Sammlerinnen auftritt, verzögert das Einsetzen des Sammelns von Ammenbienen. Durch das Einbringen zusätzlichen Ethyloleats in Dreifach Kohorten, testete ich, ob es einen direkten Einfluss auf die neuronale Plastizität der visuellen Eingangsregion des Pilzkörper Kalyx hat. Da durch die Pheromon Behandlung kein eindeutiger Verhaltensphänotyp (verzögerter Sammelbeginn) induziert werden konnte, war es nicht möglich einen direkten Zusammenhang zwischen der verstärkten Ethyloleat-Exposition und der neuronalen Plastizität des Kalyx des Pilzkörpers herzustellen. Dennoch bestätigten die Beobachtungen der synaptischen Reifung meine generellen Daten zu den natürlichen Reifungsprozessen im Kalyx des Pilzkörper.

Basierend auf dem Ergebnis, dass dendritische Plastizität einen wesentlichen Anteil an der arbeitsteilungsbezogenen neuronalen Plastizität des Kalyx des Pilzkörper hat, stellte sich die Frage, welche Proteine daran beteiligt sein könnten diese Effekte zu vermitteln. Von der

Calcium/Calmodulin abhängigen Kinase II (CaMKII) ist bekannt, dass sie speziell bei Säugetieren - aber bei Insekten (*Drosophila*, Schabe) - daran beteiligt ist, Lern- und Gedächtnisvorgänge, wie die Langzeitpotenzierung, zu ermöglichen. Neben präsynaptischen Effekten, wurde gezeigt, dass dieses Protein direkt mit Elementen des postsynaptischen Cytoskeletts interagieren kann. Als Teil meiner Dissertation habe ich das Vorkommen und die Verteilung der CaMKII analysiert. Ich konnte es hochkonzentriert in einer definierten Subpopulation der intrinsischen Pilzkörper-Neurone, den „nicht kompakten“ Kenyon Zellen, nachweisen. Das dendritische Netzwerk dieser Population verzweigt sich in zwei Kalyx Subregionen: eine olfaktorisch innervierte – die Lippe – und den Kragen, welcher optischen Eingang erfährt. Dieses Verteilungsmuster ändert sich nicht mit dem Alter oder der Aufgabe der Biene. Die hohe Konzentration von CaMKII in den dendritsichen Dornenfortsätzen und die gleichzeitige räumliche Überlappung mit f-Aktin, weisen darauf hin, dass CaMKII eine Schüsselrolle bei der Induzierung struktureller neuronaler Plastizität im Zusammenhang mit Lernen und Gedächtnisbildung und/oder Arbeitsteilung bezogener Verhaltensübergänge, zukommen könnte. Interessanterweise wies der Basalring, eine weitere Subregion des Kalyx des Pilzkörpers die dafür bekannt ist kombinierten visuellen und olfaktorischen Eingang zu erhalten und fast ausschließlich durch die „inneren kompakten“ Kenyon Zellen gebildet wird, keine Immunreaktivität auf. Dies deutet auf Unterschiede in den molekularen Mechanismen die plastische Veränderungen in den entsprechenden Kenyon zellen kontrollieren.

Wie wird die zeitliche Abstimmung der Verhaltensplastizität und neuronalen Plastizität reguliert? Für das in hohen Konzentration auf Sammlerinnen vorkommende Primer Pheromon Ethyloelat konnte durch dessen Anwendung in erhöhten Konzentrationen gezeigt werden, dass es die Verhaltensreifung durch Verzögerung des Sammelbeginns beeinflussen kann. Wie aber wird Ethyloleat transferiert und wie verschiebt es die Arbeitskräfteverteilung zwischen Innen- und Außendienst Aufgaben? Frühere Arbeiten zeigten die höchste Konzentration von Ethyloleat im Sozialmagen der Sammlerinnen und schlugen vor, dass es innerhalb der Kolonie über Trophollaxis transferiert und kommuniziert wird.

Die Ergebnisse meiner Arbeit zeigten aber eindeutig, dass Ethyloleat nicht im Inhalt des Sozialmagen und auch nicht im Regurgitat, sondern nur im Gewebe des Sozialmagens vorhanden ist. Da zusätzlich die zweithöchste Konzentration von Ethyloleat auf der Oberfläche der Kutikula von Sammlerinnen gemessen wurde, wurde Trophollaxis als Übertragungsmodus ausgeschlossen. Neurophysiologische Messungen an der Antenne

(Elektroantennografie), dem ersten olfaktorischen Neuropil (Calcium Imaging der Aktivität des Antennallobus), zeigten, dass Ethyloleat als olfaktorischer Reiz wahrgenommen und prozessiert wird. Appetitive olfaktorische Konditionierung mit Hilfe des Rüsselstreckreflexes wurde als Verhaltensparadigma verwendet um zu zeigen, dass Ethyloleat mit einer Zuckerbelohnung assoziiert werden kann. Dies deutet darauf hin, dass Arbeiterinnen in der Lage sind, die Anwesenheit dieses Pheromons zu perzipieren, zu erlernen und sich auch daran zu erinnern. Da Ethyloleat nur durch Erwärmung als Stimulus präsentiert werden konnte, lässt sich schlussfolgern, dass es über Nahbereichs/Kontakt-Chemorezeption durch das olfaktorische System wahrgenommen wird. Dies wird auch durch frühere Verhaltensbeobachtungen unterstützt.

Zusammengenommen, zeigen die in dieser Dissertation präsentierten Ergebnisse strukturelle Veränderungen in der synaptischen Architektur des Kalyx des Pilzkörpers in Zusammenhang mit Arbeitsteilung. Für das Primer Pheromone Ethyloleat, welches den Übergang von Ammendiensten zum Sammeln moduliert, zeigten die Ergebnisse eindeutig, dass es über das olfaktorische System wahrgenommen wird und vermutlich auch über diesen Weg seine Wirkung vermittelt. Dennoch konnten Manipulationsexperimente keine direkte Verbindung zwischen Ethyloleat und der synaptischen Reifung herstellen. Auf molekularer Ebene stellt CaMKII einen Topkandidaten dar, der strukturelle synaptische Plastizität im Kalyx des Pilzkörpers vermitteln kann. Eine Kombination struktureller und funktioneller Experimente ist der nächste logische Schritt um schlussendlich die Verbindung zwischen der Aktivität von Primer Pheromonen (wie Ethyloleat) und molekularen Signalwegen, die Verhaltensplastizität und synaptische Plastizität im Zusammenhang mit der Arbeitsteilung von *Apis mellifera* vermitteln, herzustellen. Die hierbei identifizierten zugrundeliegenden Prozesse werden als exzellente Modelle für ein generelles Verständnis der fundamentalen Mechanismen welche Verhaltensplastizität vermitteln, dienen.

General Introduction

Probably the most striking feature people notice while observing large colonies of eusocial hymenopteran insects, like bees, wasps or ants, is the sheer amount of animals. At a first glance virtually all individuals seem to work randomly on different tasks. With this observation in mind, the question quickly arises how social insects have become so enormous ecological successful. While only about 2 percent of the approximately 900,000 insect species on the planet are eusocial, they contribute enormously to the total animal biomass, in tropical habits up to 80% (Hölldobler and Wilson, 2008). One answer to this question clearly resides in the high level of social organization, in particular division of labor, which has evolved in social insect communities enabling the colonies to become superorganisms. In a social insect superorganism the basic functional unit are not cells but closely cooperating individuals whose overall performances cause new emergent properties at the colony level (Hölldobler and Wilson, 2008). The functional units of a honeybee colony are the morphologically different queens, drones and workers. While drones develop from unfertilized eggs, queens and workers develop from fertilized eggs (Winston, 1987). The polymorphism between the queen and worker phenotype is controlled via epigenetic mechanisms (Kucharski et al., 2008) and is induced through a prolonged feeding with royal jelly of the designated queen larvae compared to a designated worker larvae (Winston, 1987). Among those three polymorphic phenotypes division of labor can be established at several levels (see below). This allows the superorganism to express an adaptive and flexible response to environmental constraints (for review see for example Beshers and Fewell, 2001). Reproduction is restricted to queens and drones as they are, in general, the only sexually reproductive individuals with mature reproduction organs. As sexual reproduction is the predominant task of queens and drones the vast majority of tasks within the colony remain for the workers, which demands for division of labor among them. In some social insect species, for example the leaf-cutting ant Atta sexdens, workers express a pronounced polymorphism (Hölldobler and Wilson, 2008). Different environmental conditions during larval and pupal development lead to strong variations in body size of the workers, that in turn result in different behavioral repertoires (Hughes and Boomsma, 2007). In this alloethism, small “minor” workers (with head size below 0,8mm) stay inside the nest, take care of the brood and the fungus hyphae, while large “major” workers (head size up to 5mm)

forage outside the nest, cut and transport leave material (Hölldobler and Wilson, 2008). In honeybees, workers are not polymorphic and share the same morphological traits after emergence, but still have to deal with a great variety of tasks ranging from building brood cells and tending the brood to long distance flights to find profitable food sources, which requires sophisticated navigation outside the hive. In contrast to anatomical specializations, honeybees perform division of labor on the basis of a temporal polyethism, which is a major focus in this thesis.

Division of labor in honeybee colonies

Observations as early as 1609 by Charles Butler and later by various other authors (reviewed in Winston, 1987) have determined a temporal pattern as the blueprint for division of labor with workers progressing through a process of behavioral development. For approximately the first 3 weeks of adult life workers mostly stay inside the hive, while older workers become foragers and collect nectar, water and pollen outside (Lindauer, 1952; Seeley, 1982; Winston, 1987). Even though there is quite some variability and overlap, a succession of tasks that the workers go through has been revealed: starting with cleaning cells, workers move on to tending the brood and queen before becoming nectar receivers, a task that is followed by pollen handling and comb building. They then begin with ventilating and guarding tasks at the nest entrance before finally starting foraging trips collecting nectar, water and pollen. The performance of almost all these tasks is closely correlated to the presence and use of glands, which have been shown to develop and diminish during the ontogeny of an individual worker (Winston, 1987).

In particular the transition from indoor to outdoor duties represents a fascinating challenge: bees that have thus far lived in a small restricted, almost completely dark environment are then facing bright daylight and the freedom to fly for several kilometers. This brings an enormous change in olfactory, but especially visual sensory stimuli, which basically have been irrelevant before, except for short orientation flights (Capaldi et al., 2000). Furthermore, foragers need to express a completely different behavioral repertoire. This includes long distance navigation based on landmarks as well as polarized-skylight information, learning and memory tasks associated with profitable food sources and the transfer of information to nestmates via sophisticated dance communication (von Frisch, 1967). Keeping this in mind it becomes obvious that not only glands but also the central

nervous system must be a region of structural and functional changes that are needed to accommodate the transition between very different sensory environments and tasks. It can therefore be concluded that honeybee workers go through an age-related polyethism based on significant physiological adaptations.

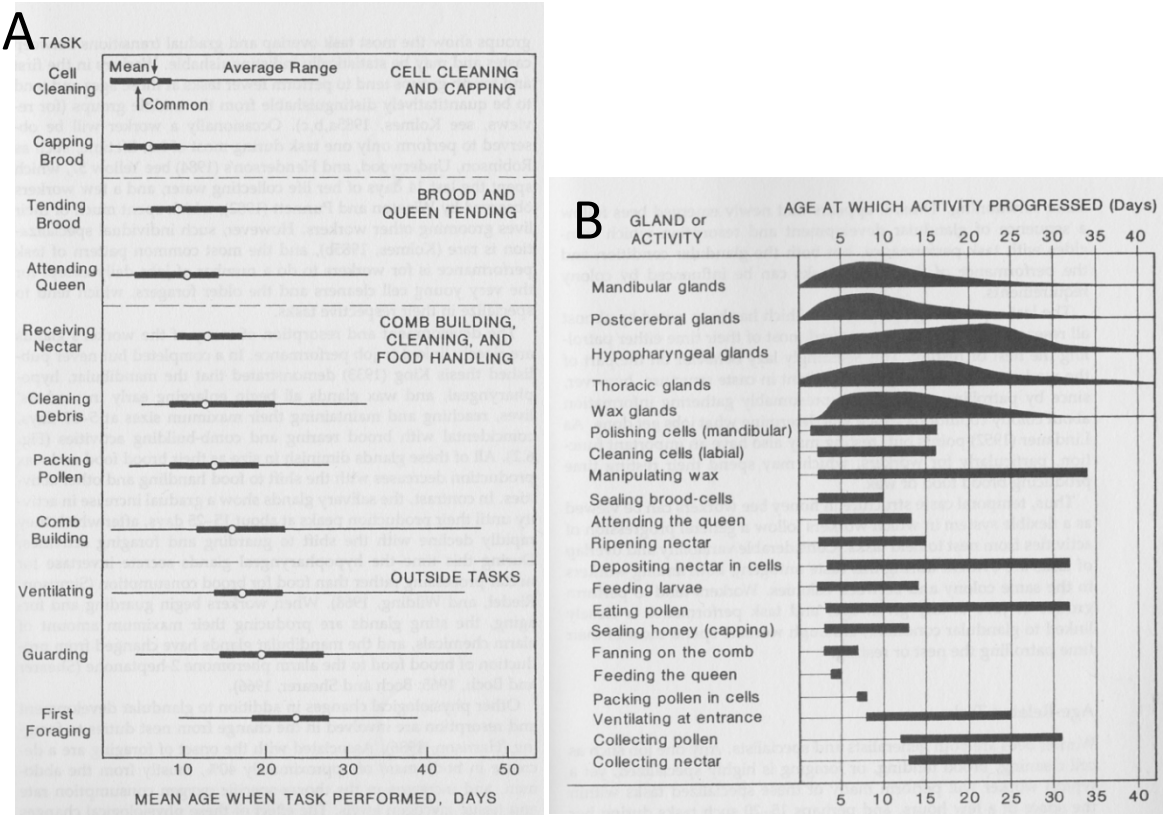


Figure 1: Task behavioral repertoire of honeybee workers and respective gland activities. **A:** The meta-analysis of 18 different datasets reveals an age-correlated succession of various in-hive tasks ending in the transition to foraging behavior. **B:** Gland activity is temporarily restricted and appears closely connected to certain tasks.

Adapted from Winston, 1987.

On the mechanistic level a major hormonal regulator of this age-related division of labor is the counteracting network of juvenile hormone and the yolk protein precursor vitellogenin (Amdam and Omholt, 2003; for review see Page et al., 2006). Juvenile hormone is released form the corpora allata (Hagenguth and Rembold, 1978; Tobe and Stay, 1985), and titers in young bees such as cell cleaners are low, but start to rise with the succession to nursing tasks, to reach their maximum just before the transition to foraging (for review see Robinson, 1992; Robinson and Huang, 1998; Bloch et al., 2002). Vitellogenin, in contrast, is produced in the fat body and levels decrease during adult maturation (Engels and Fahrenhorst, 1974). These two endocrine factors have been suggested to suppress each

other (Pinto et al., 2000; Amdam and Omholt, 2003; Guidugli et al., 2005) and, at the same time, lead to opposing effects: treatment with juvenile hormone or juvenile hormone analogue caused a precocious onset of foraging (for review see Robinson, 1992), while high vitellogenin levels inhibit the onset of foraging (Nelson et al., 2007). In the opposite direction, the removal of the corpora allata as the only known juvenile hormone source delays behavioral maturation (Sullivan et al., 2000), whereas an RNAi mediated vitellogenin knockdown leads to a precocious onset of foraging (Guidugli et al., 2005). But also other substances could be involved in regulating adult behavioral maturation. For instance biogenic amines such as serotonin, dopamine or octopamine, have been shown to alter in concentration correlated to age and task (Schulz and Robinson, 1999; Wagener-Hulme et al., 1999). For example octopamine levels are increased in foragers compared to nurses and experimental treatment of colonies with octopamine leads to an early onset of foraging (Schulz and Robinson, 2001). As a reciprocal relationship between octopamine and juvenile hormone has been described the pathways that link the two factors still need to be discovered (Schulz et al., 2002). Current evidence suggest that octopamine modulates the onset of foraging by increasing the likelihood of responding to foraging related stimuli (for review see Schulz et al., 2002; Scheiner et al., 2006).

Regulation of division of labor

The genetic background of a colony has been shown to influence to onset of foraging and could therefore represent the fundament for an internal behavioral maturation program (Page and Pankiw, 2001; for review see Page et al., 2006). But if division of labor among the workers actually was the rigid and purely age-related internal program as just suggested, honeybee colonies would be unable to quickly compensate for environmental changes when necessary. This is especially important for the transition of indoor workers to foragers as it goes along with the greatest physiological changes. In fact the work force distribution between indoor and outdoor workers can be allocated by delaying, accelerating or reverting individual behavioral development (for review see for example Robinson, 1992; Page et al., 2006; Johnson, 2010). The behavioral maturation status is reflected by corresponding juvenile hormone titers: delayed development correlates to longer duration of low juvenile hormone titers, accelerated development with a precocious rise in juvenile hormone titers, and behavioral reversion with a drop in juvenile hormone titers (Robinson et al., 1989;

Robinson, 1992; Huang and Robinson, 1996). But how is the age-related maturation process modulated? Two pheromonal systems, brood pheromone and queen mandibular pheromone, have conclusively been shown to influence division of labor. Brood pheromone, consisting of 10 components, is secreted from the larvae's salivary glands and has been shown to have releaser as well as primer effects (Le Conte et al., 1990, 2006; for review see Le Conte and Hefetz, 2008). The individual components cause various but different effects ranging from the inhibition of the worker ovaries (Mohammedi et al., 1998) to several aspects of brood care such as stimulating protein biosynthesis in the hypopharyngeal glands (Mohammedi and Crauser, 1996) or inducing workers to cap brood cells (Le Conte et al., 1990). Further, it has been shown to affect the onset of foraging - in particular pollen foraging (Pankiw et al., 1998b) – in a dose dependent manner, probably by depressing juvenile hormone titers (Le Conte et al., 2001). Queen mandibular pheromone consisting of 5 synergistically acting components, is a second multi component pheromone that has been shown to induce releaser as well as primer effects (Slessor et al., 1988; Keeling et al., 2003). It is passed within the colony by young bees which are particular attracted by it expressing the retinue behavior and will eventually get in contact with the queen (Winston, 1987). The colony wide effects range from inhibiting rearing new queens (Winston et al., 1991), suppressing ovary development of the workers (Hoover et al., 2003) to modulating comb building behavior (Ledoux et al., 2001). Similar as brood pheromone, queen mandibular pheromone has been shown to influence juvenile hormone titers, which in turn alter the age-related behavioral maturation (Kaatz et al., 1992; Pankiw et al., 1998a). In addition, queen mandibular pheromone was shown to affect dopamine pathways in the brain of young workers, which in turn have been shown to influence behavioral response thresholds (Beggs et al., 2007; Vergoz et al., 2007). A third pheromonal system contributing to the division of labor was long suspected when direct worker-worker interactions were shown as an important factor in regulating the individual behavioral ontogeny. In an early study Rösch (1930) was able to elegantly deprive a colony of all foragers and induce a precocious onset of foraging in the remaining workers. In the succession it has been revealed that deprivation of worker-worker interactions (Huang and Robinson, 1992), but especially the absence of foragers, leads to an early rise of juvenile hormone titers and, in consequence, to a precocious onset of foraging (Huang et al., 1998). From partial and complete exclusion experiments and bioassays it was possible to conclude that foragers themselves represent a

pheromone source that requires physical contact (Huang et al., 1998; Leoncini et al., 2004b; Pankiw, 2004). The identified pheromone, ethyl oleate, is highly abundant in foragers compared to workers inside the hive and was shown to delay the onset of foraging in triple-cohort colonies when artificially added (Leoncini et al., 2004a). In summary, the important information about the presence of brood, queen and foragers are mediated through pheromonal systems that have, besides other effects, at least partly been shown to prime effects on the juvenile hormone titers, thusly influencing behavioral maturation ontogeny (for review see Le Conte and Hefetz, 2008). Yet, as the overall network of factors that influence behavioral maturation is so manifold, and the linkage between the various factors is partly unknown, the actual models describing division of labor are still partly speculative (for review see for example Beshers and Fewell, 2001; Amdam and Omholt, 2003; Page et al., 2006; Johnson, 2010).

The mushroom bodies as centers of multimodal integration

In 1850 the French biologist Félix Dujardin was the first to describe prominent structures in the insect brain he named “corps pédonculés” which he assigned the ability of control over instinctive behaviour (Dujardin, 1850; reviewed in Strausfeld, 2002). These structures, apparent in all neopteran insect species (Strausfeld et al., 1998), became an important subject in insect neurobiology research and are now known as the mushroom bodies. As research progressed over the last 165 years Dujardins original assumptions have been refined and the mushroom bodies are now regarded as higher-order multi modal integration centers and are known to play a major role in learning and memory processes (for review see, for example Menzel and Giurfa, 2001; Heisenberg, 2003; Fahrbach, 2006).

Within the honeybee brain the mushroom bodies are very prominent paired structures with the protocerebrum that can be subdivided per hemisphere into two cup shaped calyces, the pedunculus, the vertical and medial output lobe (Mobbs, 1982; Fahrbach, 2006; Ito et al., 2014). These different neuropil substructures are formed by the mushroom body intrinsic neurons, the Kenyon cells, which have been first described by F.C Kenyon in 1896. With a number of about 368,000 [184,000 per mushroom body/hemisphere] (Witthöft, 1967; Strausfeld, 2002), Kenyon cells represent a large (~43%) proportion of the approximately 960,000 neurons in whole honeybee brain (Menzel and Giurfa, 2001; Rössler and Groh, 2012). They are consecutively born from neuronal precursor cells, so called neuroblasts,

during post-embryonal development in three separate, but overlapping time periods until pupal stage 6, which marks the middle of pupal development (Farris et al., 1999). During proliferation newly born Kenyon cells push older cells away from the neuroblasts leading to a distinct concentric pattern with the cells that are born latest remaining in the center (Farris et al., 1999).

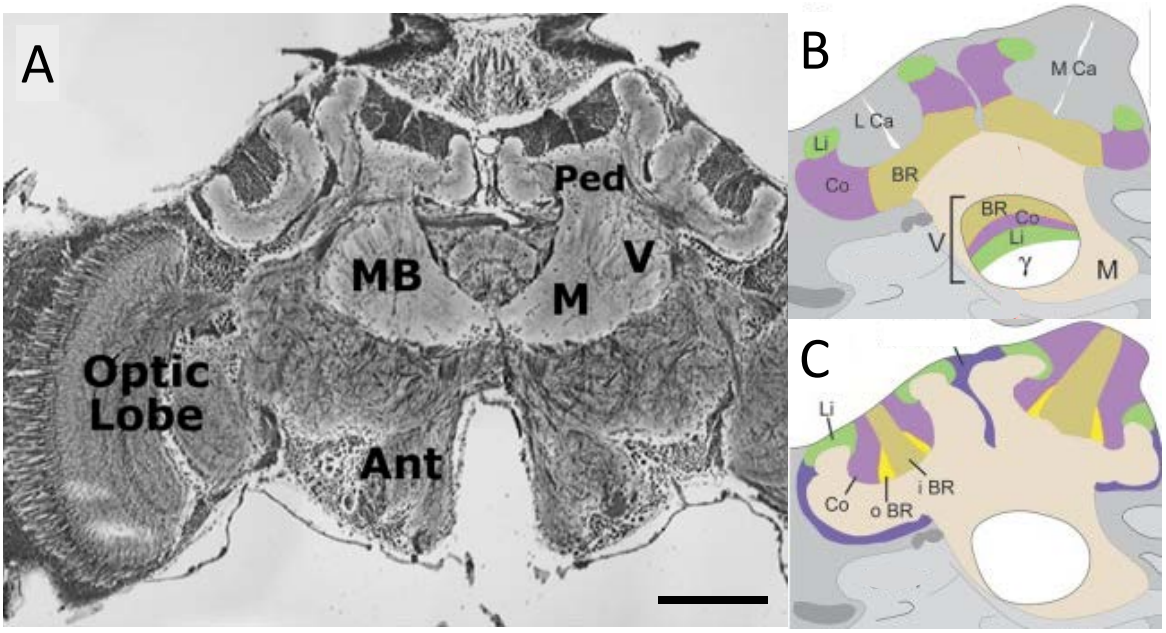


Figure 2: Neuroanatomical organization of the mushroom body. **A:** Photomicrograph of reduced silver stain depicting a panoramic overview of the honeybee brain with the mushroom bodies (MB) of each hemisphere, consisting of a lateral and medial calyx each, the pedunculus (Ped) and the medial (M) and vertical (V) output lobes. **B:** Schematized groundplan of the mushroom body with the medial and lateral calyces (M Ca, L Ca) each divided into three major subregions, lip, collar, and basal ring (Li, Co, BR). These are represented by three layers in the vertical lobe (V) laying above the fused gamma lobe (γ). The medial lobe (M) extends toward the brain's midline. **C:** Organization Kenyon cell populations indicated by the calycal region they supply: Li (lip), Co (collar), o BR, i BR (outer and inner basal ring). Scale bar = 200 µm in A

Adapted from Fahrbach, 2006 (A) and Strausfeld, 2002 (B, C).

The innermost cells of the lineage, termed "inner compact" cells, are densely packed and their cell bodies have a rather small diameter of ~4-5µm. This population forms a cone shaped region inside the calyx which is surrounded by the secondly born cell type. These so called "noncompact" cells are less densely organized, have a slightly larger cell-body diameter of ~6-7µm and fill the remaining space inside the calyx, slightly spilling over its edges. The first-born cells have a again a smaller diameter of ~4-5 µm and represent the rather small third population of Kenyon cells with just around 14,000 somata (Strausfeld, 2002), which form a layer around the calyx and are therefore called "outer compact" cells. Even though the three subpopulations differ in morphology they all share a general

“neuronal layout”: while the large dendritic arborizations compose the cup shaped calyx, the axonal projections run in a highly parallel fashion generating the pedunculus, a trunk like substructure of the neuropil that extends at the basis of the calyx. Within the pedunculus the axon bifurcate and form the two main output regions of the mushroom body, the vertical lobe (formerly named α lobe) and the medial lobe (formerly named β lobe) (Mobbs, 1982; Farris et al., 1999; for review see for exmaple Fahrbach, 2006; Ito et al., 2014).

Being largely composed of the Kenyon cell dendritic arborizations the calyx represents a major input region of the mushroom body. It can be further subdivided into the lip and collar, both formed by the dendrites of the noncompact cells, and the basal ring, which contains the dendritic network of the inner compact cells (Mobbs, 1982). The outer compact cells diffuse their branches in in the whole calyx contributing to all three subregions (Mobbs, 1982; Strausfeld, 2002). All three subregions have a similar synaptic architecture in common with distinct synaptic complexes, so called microglomeruli. These characteristic microcircuits have been first described at the ultrastructural level by Trujillo-Cenóz (1962) and Steiger (1967). Each microglomerulus comprises a central axonal bouton of a sensory projection neuron, which is surrounded by dendritic profiles originating from the Kenyon cells (Ganeshina and Menzel, 2001; Frambach et al., 2004; Groh et al., 2004). Additionally, few arborizations from γ-aminobutyric acid (GABA) (Ganeshina and Menzel, 2001; Grünewald, 1999), octopamine (Hammer, 1993; Kreissl et al., 1994) or dopamine (Blenau et al., 1999) positive extrinsic neurons contribute to the microglomerulus microcircuits. Only in rare cases, GABAergic neurons may form central boutons themselves (Ganeshina and Menzel, 2001).

The differentiation between the three calyx subregions is not only characterized by its anatomical architecture but also because of the sensory input each subregion processes. The lip exclusively receives input from olfactory projection neurons, while the collar processes visual information, and the basal ring is innervated from both modalities (Mobbs, 1982; Gronenberg, 2001; Ehmer and Gronenberg, 2002; Farris and Sinakevitch, 2003; Kirschner et al., 2006). Additionally, mechanosensory as well as gustatory input from the subesophageal ganglion is conveyed via the subesophageal-calycal tract to a small area in the upper part of the collar and in the basal ring (Schröter and Menzel, 2003; Farris, 2005). The olfactory information originates from the ipsilateral antennal lobe and is relayed to the lip and basal ring via two distinct tracts of projection neurons (Mobbs, 1982; Abel et al., 2001). Depending

on the antennal lobe glomerulus the projection neuron originates, it either runs via the medial antennal-lobe tract or the lateral antennal-lobe tract to the calyx (Kirschner et al., 2006; Ito et al., 2014). Projection neurons of the two antennal lobe output tracts have recently been shown to perform parallel processing by extracting different attributes from similar odorant stimuli, roughly quality (medial antennal-lobe tract) and temporal information (lateral antennal-lobe tract) (Brill et al., 2013; for review see Rössler and Brill, 2013). Within the mushroom body calyx the medial antennal-lobe projection neurons branch across the entire lip region with the highest densities in the cortical (outer) zones, and in the peripheral part of the basal ring. The lateral antennal-lobe tract projection neurons, in contrast, exclusively innervate the central core of the lip and a more central region of the basal ring (Kirschner et al., 2006.) This spatial separation of projections indicates further segregated olfactory processing. A similar assumption on segregated processing seems likely for visual information, which originates in the ipsi- and contralateral optic lobes and is transmitted to the collar and basal ring via the anterior–superior optic tract, the anterior–inferior optic tract, and the lobular tract (Ehmer and Gronenberg, 2002). Similar to the olfactory regions of the calyx the projection neurons express a strong local representation with the medulla projection neurons terminating in alternating layers within the lateral part of the collar and lobula projection neurons forming a similar layered pattern in the medial part of the collar (Ehmer and Gronenberg, 2002; Paulk and Gronenberg, 2008). In the basal ring visual information is processed in the more outer part, clearly separated from the olfactory innervated central regions. Within this optical region lobula neurons, in general, are more frequent, but clearly separated from the medulla projection neurons, which are restricted to a small distal area (Ehmer and Gronenberg, 2002). Different qualities of the visual stimuli such as chromatic, temporal or motion information, therefore, seem to be processed in segregated layers of the collar (Paulk and Gronenberg, 2008; Paulk et al., 2009).

This separation of the sensory input is maintained when the bundles of axonal projections of the Kenyon cell run through the pedunculus and form distinct layers in vertical output lobe of the mushroom body. Strausfeld (2002) distinguishes four layers: The most dorsal first layer corresponds to the basal ring (inner compact cells), the second layer corresponds to the collar (noncompact cells), the third layer contains the axons of noncompact Kenyon cells forming the lip and the fourth layer arises from the outer compact cells and resembles the γ-lobe often found separately in other insect species (Mobbs, 1982; Strausfeld, 2002). From

the vertical and medial lobes about 400 extrinsic output neurons transmit information to other regions of the protocerebrum (Mobbs, 1982; Rybak and Menzel, 1993).

Structural plasticity of the mushroom bodies

As described earlier, division of labor within the female honeybee worker caste comprises an age-related polyethism. The succession of tasks inside the hive, especially the transition from nursing duties to foraging tasks outside, not only goes along with tremendous changes in the sensory environment but also requires completely different behavioral repertoires. These challenges have to be met by adaptive adjustments of the sensory systems and higher-order integration centers in the brain. Besides the antennal lobes (Winnington et al., 1996; Sigg et al., 1997; Morgan et al., 1998; Brown et al., 2002, 2004; Hourcade et al., 2009; Arenas et al., 2012), the mushroom bodies have most extensively been investigated for structural changes associated with age, foraging behavior, learning and memory processes, social interaction and sensory experience. Various studies have revealed that the mushroom bodies, in particular the mushroom body calyces, undergo a substantial volume increase during the lifetime of a honeybee (for review see Fahrbach and Dobrin, 2008). Those studies have used a broad range of experimental approaches like age-matched cohorts, sensory deprivation or induction of precocious onset of foraging to uncover that the observed volume increase is age-, task-, and experience-related (Withers et al., 1993, 1995; Durst et al., 1994; Fahrbach et al., 1998; Farris et al., 2001; Groh et al., 2012; Ismail et al., 2006). For example, even light deprived honeybees express a volume increase during the first week after eclosion (Fahrbach et al., 1998), and age-matched experienced foragers have a larger calyx volume compared to the control group (Farris et al., 2001). It has, therefore, been concluded that the observed volume increase consists of at least two independent components, an "experience-expectant" internal program and a sensory stimuli driven "experience-dependent" volume increase (Fahrbach et al., 1998). Similar results have been obtained for other social Hymenoptera like the carpenter ant *Camponotus floridanus* (Gronenberg et al., 1996), the desert ant *Cataglyphis bicolour* (Kühn-Bühlmann and Wehner, 2006), and the paper wasp *Polybia aequatorialis* (O'Donnell et al., 2004). What are the underlying mechanisms that lead to these well described volume increases? Proliferation of Kenyon cells (or projection neurons) can be ruled out, as adult neurogenesis was shown to be absent in the honeybee brain (Fahrbach et al., 1995; Farris et al., 1999; Ganeshina et al., 2000). The

dendritic and axonal network of the calyx, however, offers a great potential for structural changes and is accessible for quantification at the cellular and subcellular levels. Farris et al. (2001) were already able to show that an outgrowth of the Kenyon cell dendritic network correlates with foraging experience, consequently leading to an increased calyx volume. The synaptic architecture of the mushroom-body calyx into microglomerular synaptic complexes offers an ideal substrate for investigating structural neuronal plasticity. The dendritic profiles surrounding the presynaptic boutons of the projection neurons terminate in f-actin rich dendritic spines, that form the synaptic contacts with the projection neuron boutons (Farris et al., 2001; Ganeshina and Menzel, 2001; Yasuyama et al., 2002; Frambach et al., 2004; Groh et al., 2012). Such dendritic spines, first described by Ramón y Cajal in 1891 in the mammalian Purkinje cells, are known to be highly motile and plastic, thus promoting structural adaptations of the postsynapse (Yuste and Denk, 1995; Kaech et al., 2001). The same holds true for the Kenyon cell dendritic spines which have early on been identified to express plastic changes correlated to age (Coss et al., 1980) and sensory exposure (Brandon and Coss, 1982). Several studies in the honeybee (Krofczik et al., 2008; Hourcade et al., 2010; Groh et al., 2012; Scholl et al., 2014) and in *Cataglyphis* desert ants (Seid and Wehner, 2009; Stieb et al., 2010, 2012) have revealed that mushroom body calyx microglomeruli are sites of structural synaptic plasticity. Interestingly, it appears that maturation as well as the exposure to light caused pruning of microglomeruli (Seid and Wehner, 2009; Stieb et al., 2010, 2012; Groh et al., 2012; Scholl et al., 2014), while learning and memory processes lead to the formation of new microglomeruli (Hourcade et al., 2010).

The molecular mechanisms initiating and maintaining neuronal plasticity in the mushroom-body are largely unknown. However, it has been established, that pharmacological stimulation of muscarinic–type cholinergic receptors is sufficient to mimic foraging-related sensory exposure and induce an outgrowth the Kenyon cell dendritic network (Ismail et al., 2006; Dobrin et al., 2011). Dobrin and Fahrbach (2012) further show that the activity of Rho GTPases, small monomeric G proteins, which are involved in the reorganization of the neuronal cytoskeleton, is correlated to age and foraging experience suggesting them as an effector that could at least partly mediate experience-dependent neuronal plasticity. Also in the direct context of learning and memory processes, neurotransmitters - like acetylcholine, glutamate, octopamine, dopamine or serotonin - have been shown to modulate associative learning (e.g. Hammer and Menzel, 1998; Lozano et al., 2001; Locatelli et al., 2005; Wright et

al., 2010; for review see Giurfa and Sandoz, 2012), which in turn has been demonstrated to induce structural synaptic plasticity (Hourcade et al., 2010). It is therefore not unlikely that many transmitters regulate experience-induced structural changes in the honey bee brain and are linked to the cytoskeleton via the Rho GTPases (Dobrin and Fahrbach, 2012). Another likely candidate for inducing plastic changes in calyx is the calcium calmodulin depenent protein kinase II (CaMKII), which is highly concentrated in the noncompact Kenyon cells (Kamikouchi et al., 2000). This protein is well known for its role in learning processes and long-term memory formation (for review see Lisman et al., 2002; Mayford, 2007) in vertebrates (Malenka et al., 1989) as well as invertebrates (Lent et al., 2007) including the honeybee (Matsumoto et al., 2014). As it partly shows direct interaction with cytoskeletal proteins, inducing changes in spine size and shape (Mayford, 2007; Abraham, 2008; Okamoto et al., 2009), this protein could be a key player transmitting long-term memory effects on structural plasticity as shown by Hourcade et al. (2010).

Thesis outline

Division of labor among honeybee workers is based on an age-related polyethism that in turn relies on behavioral maturation processes that are particular interesting during the switch from indoor to outdoor duties. This transition involves tremendous changes in the sensory environment and eventually demands for a completely different behavioral repertoire. As the mushroom body neuropil is not only a very prominent multimodal integration center that is involved in learning and memory tasks but is also anatomical well described, the honeybee represents an excellent model to study underlying mechanism of neuronal plasticity.

The present thesis aims to link division of labor in honeybee workers with structural plasticity in the mushroom body calyx focusing on the following questions:

- How is the synaptic architecture affected during behavioral maturation? Is it primarily correlated to an age-dependent internal program, or can it be adjusted in a flexible manner?
- What are the cellular mechanisms involved in neuronal (volume) plasticity of the mushroom body calyx?
- Are effects of external behavioral maturation influencing factors, such as primer pheromones, reflected in neuronal plasticity processes of the mushroom body?
- Is the protein calcium/calmodulin-dependent kinase II a relevant protein for structural neuronal plasticity in the honeybee brain?
- How is information about the primer pheromone ethyl oleate distributed within the honeybee colony?

Correspondingly the research section of this thesis is organized in three chapters addressing the following aspects:

1) *Structural plasticity in the mushroom body calyx*

   The mushroom bodies as centers of multimodal integration as well as learning and memory processes have been established as a region that expresses neuronal plasticity, in particular a neuropil volume increase correlated to age, task and experience. In this study the underlying mechanisms at the level of the synaptic architecture were investigated in great detail to establish a framework for further

manipulative approaches that aim to disentangle the chain of events that connects division of labor to neuronal plasticity. In this context the role of the behavioral maturation influencing primer pheromone ethyl oleate was investigated and correlated with the changes in the synaptic organization of the mushroom body calyx.

2) *CaMKII - a key protein mediating structural plasticity of mushroom body calyx*?
   Calcium/calmodulin-dependent kinase II (CaMKII) is a protein that has been shown to be involved in neuronal plasticity processes in various animal models. In this study the occurrence and distribution of CaMKII in the honeybee brain with special focus on the mushroom body and its synaptic architecture was investigated. Furthermore, possible changes in the distribution pattern of CaMKII during age-related behavioral maturation were explored that could point towards a specific role of CaMKII in mediating structural plasticity related to division of labor.
3) *Transmission mode of the primer pheromone ethyl oleate*
   The forager produced primer pheromone ethyl oleate was shown to delay behavioral maturation in workers and by this the onset of foraging. As highest concentrations were found in the foragers' honey crop, it was suggested that the pheromone is distributed in the colony via trophallaxis. In this study the distribution of ethyl oleate on the body of foragers was revisited and concentrations were analyzed in more detail. Olfaction was investigated as a possible mode of transmission using three different methods to prove that ethyl oleate is received as an olfactory stimulus – electroantennogram recordings to test whether ethyl oleate is received by antennal receptors; calcium imaging of glomerular activities in the antennal lobe to test whether it is processed in the primary olfactory centers; and appetitive olfactory conditioning to test whether it is perceived as behaviorally relevant information that could influence division of labor.

All three chapters are based on publications in peer-reviewed journals (one is still under revision) and are each organized according to scientific publication standards with an abstract, introduction, material and methods, results and discussion section. A general introduction, giving a broad overview on relevant topics, and a general discussion, integrating the results of each chapter, constitute the framework of this thesis.

Chapter I

Neuronal plasticity in the mushroom-body calyx during adult maturation in the honeybee and possible pheromonal influences

Abstract

Honeybee workers express a pronounced age-dependent polyethism switching from various indoor duties to foraging outside the hive. This transition is accompanied by tremendous changes in the sensory environment that sensory systems and higher brain centers have to cope with. Foraging and age have earlier been shown to be associated with volume changes in the mushroom bodies (MBs). Using age- and task-controlled bees this study provides a detailed framework of neuronal maturation processes in the MB calyx during the course of natural behavioral maturation. We show that the MB calyx volume already increases during the first week of adult life. This process is mainly driven by broadening of the Kenyon cell dendritic branching pattern and then followed by pruning of projection neuron axonal boutons during the actual transition from indoor to outdoor duties. To further investigate the flexible regulation of division of labor and its neuronal correlates in a honeybee colony, we studied the modulation of the nurse-forager transition via a chemical communication system, the primer pheromone ethyl oleate (EO). EO is found at high concentrations on foragers in contrast to nurse bees and was shown to delay the onset of foraging. In this study EO effects on colony behavior were not as robust as expected, and we found no direct correlation between EO treatment and synaptic maturation in the MB calyx. In general we assume that the primer pheromone EO rather acts in concert with other factors influencing the onset of foraging with its effect being highly adaptive.

This chapter is based on a pre-edited manuscript:

Muenz TS, Groh C, Maisonnasse A, Le Conte Y, Plettner E, Rössler W. 2015. Neuronal plasticity in the mushroom-body calyx during adult maturation in the honeybee and possible pheromonal influences. under revision

Introduction

In colonies of the European honeybee (*Apis mellifera*) with up to 60,000 individuals, the female worker caste faces an enormous variety of different tasks that are necessary to ensure the survival of the colony (Winston, 1987). Worker bees not only have to take care of the brood inside the nest, build and maintain the complex nest structure and climatic conditions, but also have to regulate food intake depending on the needs of the colony. Workers accomplish this variety of tasks via a pronounced age-related polyethism resulting in division of labor. In the first three weeks of adult life worker bees are engaged with various indoor duties, while older workers normally become foragers and start to collect nectar, pollen and water (Rösch, 1925; for review see Johnson, 2010). With the switch from indoor to outdoor duties workers have to solve a completely different set of challenges like visual orientation, long distance flight navigation, learning of profitable food source locations, information transfer via dance communication to other bees, and olfactory learning and memory tasks associated with foraging (Robinson, 1992; Menzel, 2001). Environmental cues completely change along with this behavioral transition and need to be accommodated by adaptive adjustments in sensory systems and higher-order integration centers in the brain.

A likely substrate for such plastic changes in the neuronal circuitry are the mushroom-bodies (MBs), prominent centers in the honeybee brain (Fig. 1) that were shown to be involved in higher sensory integration as well as learning and memory processes (Menzel, 1999; Giurfa, 2007; Hourcade et al., 2010; Rössler and Groh, 2012). In the honeybee, the MBs comprise ~184,000 densely packed intrinsic neurons per hemisphere, the Kenyon cells (KCs) (Kenyon, 1896; Witthöft, 1967; Strausfeld, 2002). Large dendritic arborizations of KCs mainly shape the MB calyces, paired cup-shaped structures representing major sensory-input regions of the MBs (Mobbs, 1982; for review see Fahrbach, 2006). One MB calyx can further be divided into three subdivisions (Fig. 1), the lip receiving olfactory input from the antennal lobes, the collar innervated by visual projection neurons from the optic lobes, and the basal ring receiving input from both modalities (Mobbs, 1982; Gronenberg, 2001; Ehmer and Gronenberg, 2002; Farris and Sinakevitch, 2003; Kirschner et al., 2006). The MB collar is further structured into a loose and dense synaptic region (Fig. 1 B-D, see also Groh et al., 2004) receiving specific chromatic and motion information from the optic lobula or medulla

(Ehmer and Gronenberg, 2002; Paulk and Gronenberg, 2008). The MB calyx receives further input from the subesophageal tract in a small band between the lip and collar region and in the basal ring, most likely representing gustatory and mechanosensory input from the proboscis (Schröter and Menzel, 2003). All MB calyx subregions have a similar synaptic architecture with distinct synaptic complexes called microglomeruli (MG) in common (Ganeshina and Menzel, 2001; Frambach et al., 2004; Groh et al., 2004). MG were described for the first time in beetles and ants (Trujillo-Cenóz and Melamed, 1962; Steiger, 1967). Each MG comprises a large presynaptic bouton, that is an axonal terminal of visual or olfactory projection neurons, which are embedded in many f-actin rich postsynaptic profiles, most of them originating from KC dendrites (Groh et al., 2004, 2006, 2012; Frambach et al., 2004) plus few from γ-aminobutyric acid (GABA) (Ganeshina and Menzel, 2001 Grünewald, 1999), octopamine (Hammer, 1993; Kreissl et al., 1994) or dopamine (Blenau et al., 1999) positive extrinsic neurons. The highly parallel axonal projections of the KCs form the MB peduncle and bifurcate into the horizontal and vertical lobes (Mobbs, 1982; Ito et al., 2014).

Most studies that investigated neuroanatomical changes associated with the switch from indoor to outdoor duties were based on volumetric measurements and mainly show that the MB calyces undergo a substantial volume increase during this transition (for review see Fahrbach and Dobrin, 2008). Using sophisticated experimental manipulations such as age-matched cohorts, induction of precocious onset of foraging, or sensory deprivation revealed that the observed volume increases are age-, task-, and experience-dependent (Withers et al., 1993; Durst et al., 1994; Fahrbach et al., 1998; Ismail et al., 2006). A recent ultrastructural study revealed that the MB-calyx volume increase between nurse bees and foragers is accompanied by an overall reduction of the number of MG - at the same time there is a significant increase in postsynaptic contacts and structural changes in active zones of individual synaptic boutons indicating an overall increase in synaptic strength and divergence (Groh et al., 2012). The reduction in MG numbers can be assigned to a pruning of projection-neuron boutons, while the increase of postsynaptic profiles supports previous findings of a pronounced outgrowth and the increase in the dendritic branching pattern of KCs (Farris et al., 2001).

The transition from nurse bees to foragers is not strictly age-dependent, but instead rather flexible to adjust the indoor/outdoor work-force distribution as a function of the actual needs of the colony (Lindauer, 1952; Robinson, 1992). In addition to the evidence that queen

mandibular pheromone (QMP) (Pankiw et al., 1998a) and brood pheromone (Pankiw et al., 1998b; Le Conte et al., 2001) influence the onset foraging activity, worker-worker interactions were shown to be an important factor in regulating the individual foraging ontogeny. This goes back to the observation that young nurse bees in a forager-deprived colony start precocious foraging (Rösch, 1930). Several studies have narrowed down that foragers represent a pheromone source themselves that regulates the onset of foraging in nurse bees (Huang et al., 1998; Leoncini et al., 2004b; Pankiw, 2004). The identified pheromonal substance was ethyl oleate (EO), which is highly abundant in foragers compared to nurse bees and was used to experimentally delay the onset of foraging in triple-cohort colonies (Leoncini et al., 2004a). Recent studies show that EO is mainly located on the cuticular surface, and consequently sensed and processed via the olfactory system (Muenz et al., 2012). Furthermore, biosynthetic studies have shown that EO is produced by lipases expressed in the esophagus and honey crop of foragers, by condensation of oleic acid and ingested ethanol (Castillo et al., 2012a). EO produced in the gut is then transported to the cuticle by a methoprene-inducible, as yet unknown transport mechanism (Castillo et al., 2012b).

Based on these previous studies on the behavioral transition from nurse bees to foragers and the associated changes in the MBs, the goal of the present study was to analyze and quantify changes in the neuroarchitecture of the MB calyx over the course of natural adult maturation on a 3D basis and to relate them to MB volume changes over the course of behavioral maturation. Furthermore, we ask how different aspects of MG reorganization are correlated to the onset of foraging and whether these changes in neuroarchitecture are influenced by alterations of the primer pheromone EO concentration within the colony.

Material and Methods

Synaptic maturation in the MB calyx was studied under natural rearing conditions using age-cohort experiments within normal colonies kept according to standard commercial practice, while the influence of EO on synaptic plasticity was investigated using triple-cohort experiments.

Bees and cohort experiments

Age-cohort experiments

Age-cohort experiments were conducted using *Apis mellifera carnica* from the institutional apiary at the University of Würzburg, Germany. As described in Groh et al. (2012) each cohort consisted of around 500 age-synchronized bees (eclosion period of 12 hours) that were paint marked on the thorax directly after emergence and returned to their host colony headed by a single mated queen. To follow natural maturation of the MB calyx, a group of bees was dissected right after paint marking (1-day-old), while the other groups of these age cohorts were collected from inside the hive on days 7, 15. Bees on day 32 were caught at the hive entrance and identified as foragers by their pollen loads.

Triple-cohort experiments

During summer season of 2008 and 2009 triple-cohort experiments were conducted in pairs (treatment and control) using *Apis mellifera ligustica* from the apiary of the INRA institute "Abeilles et Environment" in Avignon, France as this subspecies has been shown to express the strongest behavioral response to EO (Brillet et al., 2002). As described earlier by Leoncini et al. (2004a) each cohort consisted of 3 groups (1-day-old paint marked bees [focal group], nurse bees and foragers) of 500 individuals each to roughly simulate a natural hive demography (Giray and Robinson, 1994), hosted in small hive boxes with two frames (one with honey and one empty). All bees in each pair (see next section) were derived from the same host colony to control for the genetic background. As QMP (Pankiw et al., 1998a) and BP (Pankiw et al., 1998b; Le Conte et al., 2001) are known to influence maturation and the onset of foraging, cohorts received one plastic strip of commercially available QMP blend (Bee Boost, PheroTech, Vancouver, Canada) instead of a live queen and were kept brood-free this way. Strips release one queen equivalent QMP per day and were replaced after 14 days. For neurohistological procedures to analyze MG densities in the MB calyx, 12-15 focal bees were removed from each of the triple-cohort colonies on days 7 and 15. The information whether bees belonged to the EO treatment or control group was blinded during all steps of bee collection, tissue preparation and neuroanatomical analysis.

Pheromone treatment and behavioral observations

Similar to Leoncini et al. (2004a) 6 pairs of triple-cohort colonies were established to directly compare the influence of EO on behavioral and synaptic maturation. Every 24 hours treatment colonies received 1 g sugar candy (30% honey, 70% powdered sugar) containing 2.1 mg EO (Sigma-Aldrich, France), while control cohorts received 1 g pure sugar candy. No trace of sugar candy was found after 24 hours leading to the assumption of a complete uptake and spread of food throughout the entire colony (Leoncini et al., 2004a).

Starting 5 days after setting up the triple-cohort colonies, 4 daily observations between 9am and 4pm (peak time of flight activity) were performed to evaluate the effect of EO on the behavioral maturation rate by determining the mean age at which the first 50 bees of each group started to forage. For this the hive entrance was blocked for 20 min (5 min accommodation phase, 15 min observation phase) with a metal screen leading to an accumulation of returning bees on the screen. All returning bees of the focal group that were identified as foragers by their swollen abdomen or pollen loads received an additional paint mark and were registered. A census count of all focal bees on the two frames of each colony was performed when the experiments were finished (3-4 weeks) to determine whether a substantial loss of focal bees had occurred (data not shown). Behavioral observations as well as daily exchanges of sugar candy were performed blinded towards the information of which colony received EO treatment.

Neuroanatomical procedures

For all following neuroanatomical procedures bees were anaesthetized on ice and mounted for preparation. During subsequent brain dissections heads were covered with physiological saline (130 mM NaCl, 5 mM KCl, 4 mM MgCl2, 5 mM CaCl2, 15 mM Hepes, 25 mM glucose, 160 mM sucrose; pH 7.2) and a rectangular window was cut between the compound eyes, ocelli and the basis of the antennae to remove glands, tracheae and the pharynx. Brain tissue was immediately transferred into ice-cold 4% formaldehyde (FA; methanol free, 28908, Fischer Scientific, Schwerte, Germany) in 0.01 M phosphate-buffered saline (PBS; pH 7.2) and fixated at 4°C overnight.

Neuronal tracing

For mass fills of dendritc KC arborizations a modified protocol from Kirschner et al. (2006) was applied (for details see there). Using the broken tip of a glass electrode an Alexa Fluor 488 dextran, 10,000 MW, lysine-fixable (D 22910; Molecular Probes, Eugene, USA) crystal was punctuated into the vertical lobe of mounted living bees with opened head capsule. Bees were kept alive for three hours in humid chambers to allow the tracer to diffuse to the calyx before brains were dissected and further processed as agarose preparations (see below). In this experiment a primary mouse anti β-tubulin antibody (1:2000; MAB3408, Chemicon, Merck Millipore) in combination with Alexa Fluor 568 conjugated goat anti-mouse antibody (A-11004, Molecular Probes, Eugene, USA) was used to label tubulin rich fibres.

Wholemount preparations

A recently established protocol (Groh et al., 2012) was used to label presynaptic terminals in the MB calyx of the age-controlled bees in the age-cohort experiments. Fixated brains were treated with PBS and PBS Triton X-100, before being incubated as wholemount preparations with the primary mouse antibody SYNORF1 (1:10; kindly provided by E. Buchner, University Würzburg, Germany) labeling the vesicle associated protein synapsin I (detailed antibody characterization in Pasch et al., 2011). For fluorescence labeling an Alexa Fluor 488 conjugated goat anti-mouse antibody (1:250; A-11001, Molecular Probes, Eugene, USA) was applied before brains were dehydrated in an ascending ethanol series and mounted on custom made metal slides in methyl salicylate (M-2047, Sigma Aldrich, Steinheim, Germany).

Agarose sections

Pre- and postsynaptic compartments of MGs in the MB calyx of 7- and 15-day-old bees from the triple-cohort experiments and age controlled bees from the age-cohort experiment were visualized according to Stieb et al. (2010) (for details see there). Fixated brains were washed with PBS before embedded in Agarose (Agarose II, no. 210-815, Amresco, Solon, OH) and sectioned in frontal planes at 100µm. Sections were incubated with Alexa Fluor 488 conjugated phalloidin (0.2 units, A12379, Molecular Probes, Eugene, USA) to mark f-actin. For double labeling sections were additionally incubated either with the primary mouse antibody SYNORF1 (1:10) to visualize vesicle associated synapsin or with a primary mouse anti α-tubulin antibody (1:500; CP06, Calbiochem, Merck Millipore) to identify α-tubulin. For

fluorescence labeling an Alexa Fluor 568 conjugated goat anti-mouse antibody (1:250; A-11001, Molecular Probes, Eugene, USA) was applied before sections were mounted on slides in 80% glycerol in PBS.

Laser scanning confocal microscopy, image processing, and data analysis

All histological preparations were visualized using a laser-scanning confocal microscope (Leica TCS SP2 AOBS, Leica Microsystems AG, Wetzlar, Germany) equipped with an argon/krypton and 3 diode lasers. Three HC PL APO objective lenses (10x/0.4 NA imm; 20x/ 0.7 NA imm and 63x/1.20 NA imm) with additional digital zoom were used for image acquisition.

Whole mount preparations of synapsin labeled brains from the age-cohort experiments were scanned as two stacks (Groh et al., 2012): an overview stack (5 µm step size) through the entire medial calyx of the left hemisphere for neuropil volume measurements and a high resolution stack (0.5 µm step size) of about 10 µm within the corresponding lip and dense collar region (Fig 1B, C) to allow for synaptic density quantification. Image processing was performed using 3D image software AMIRA 5.3 (FEI Visualization Group, Mérignac, France). Calyx volume measurements were based on outline and interpolation reconstructions of the 4 different calyx subregions: lip, basal ring, loose and dense collar (Fig. 1D). Synaptic density was measured in 3D by counting synapsin positive profiles in defined regions of interest (cubic volumes of 1000 µm³ (Fig. 1E)) applied to the lip and dense collar subregion: four volumes per lip region (Fig. 1B, ROI 4-7), and three volumes per dense collar region (Fig. 1B, ROI 1-3). Synaptic density measurements for each region of interest were averaged per individual and pooled for the different age groups. Estimations of the overall MG numbers per calyx were obtained by extrapolating the mean density of boutons to the respective reconstructed volume.

In agarose sections single optical images of the medial calyx of the left hemisphere were taken at a defined central plane identified by clearly visible anatomical landmarks: visible peduncle, MB medial lobe, upper and lower unit of the central complex (Fig. 1A, B). All double labeled preparations including synapsin/f-actin, α-tubulin/f-actin, neuronal tracing/β-tubulin were scanned as individual channels and further processed in FIJI (ImageJ 1.44c; Wayne Rasband, National Institutes of Health, Bethesda, MD). 2D synaptic density quantifications of brains from the paired triple-cohort experiments were performed in close

analogy to Stieb et al. (2010) by counting synapsin positive profiles surrounded by f-actin phalloidin positive profiles in three defined circular areas of 400µm² (Fig. 1C) that were applied to the dense collar region (Fig. 1B, ROI 1-3). Synaptic density measurements of each circular area were averaged per individual and pooled for the different age and treatment groups.

Statistical analysis and graphical editing

All statistical analyses (see details in the results section) were performed with SPSS 15.0 software (SPSS, Chicago, USA). Graphs and figures were edited by using Corel Draw X6 (Corel, Ottawa, Canada) and, in some cases, adjusted for brightness and contrast.

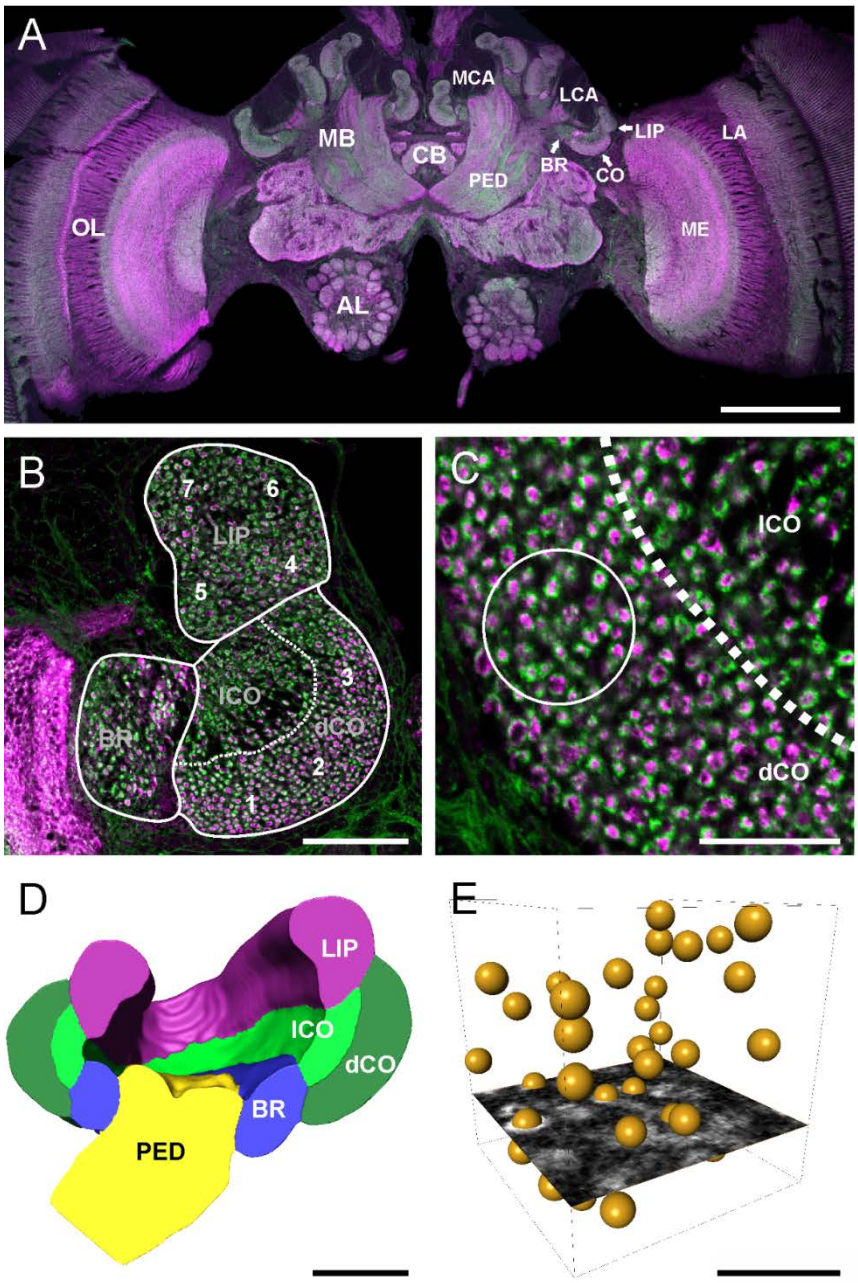


Figure 1. Overview images and 3D reconstructions of the honeybee brain. All fluorescence images are labeled for synapsin (magenta) and f-actin (green) **A**: Panoramic frontal plane overview of the honeybee brain with indication of prominent neuropils. **B**: Magnification of one "branch" of the medial calyx in frontal section showing its subregions innervated by different sensory modalities. Synaptic density measurements were performed at the indicated regions of interest (1-7); ROI 1-7 used for 3D analyses, ROI 1-3 used for 2D analyses. **C:** High magnification of the transition zone between loose and dense collar revealing the common microglomerular synaptic layout of the mushroom-body calyx: synapsin positive projection neuron boutons surrounded by f-actin rich Kenyon cell dendritic spines. White circle exemplarily illustrates 400 µm² used for 2D synaptic density measurements. **D:** Frontal section through a 3D reconstruction of the mushroom body calyx based on synapsin labeled wholemount preparations. **E:** Exemplary 1000 µm³ volume box with a single optical sections of synapsin labeled boutons used for 3D synaptic density measurements. Spheres (yellow) serve as landmarks for individual boutons. AL: antennal lobe, BR: basal ring, CB: central body, CO: collar, dCO: dense collar, LA: Lamina, LCA: lateral calyx, LIP: lip, lCO: loose collar, MB: mushroom-body, MCA: medial calyx, ME: medulla, OL: optical lobe, PED: pedunculus. Scale bars = 400 µm in A; 50 µm in B; 20 µm in C; 100 µm in D; 5 µm in E.

Results

Structural plasticity in the mushroom-body calyx during adult maturation

To analyze the natural time course of synaptic maturation processes in the MB calyx, we used synapsin labeled wholemount preparations of defined consecutive age groups from our age-cohort experiments. Treatment with synapsin antibodies produced a clearly visible staining throughout the entire depth of the calyx that enabled us to reconstruct the calyx three-dimensionally from low magnification scans. High magnification allowed quantification of the density of individual large synapsin positive presynaptic terminals that could be extrapolated to the volumes of the MB calyx subdivisions (Fig. 1). This approach provides a framework for studies investigating perturbations of normal maturation.

Qualitative comparison of individual optical sections taken at the same depth indicated an overall neuropil volume increase of the MB calyx in the course of natural maturation (Fig. 2A-D). At the quantitative level, we measured the volume of all four calyx subregions separately, the lip, basal ring, and the loose and dense collar (Fig. 1B, D and Table 1), but mainly focused on the lip and the dense collar for statistical analyses. The dense collar is identifiable as a distinct and clearly defined region of the collar with a high density of synapsin positive boutons (Groh et al., 2012). The lip and the dense collar are particular interesting as they receive different sensory input (lip: olfactory, dense collar: visual) and are most accessible for quantitative synaptic analyses (see below).

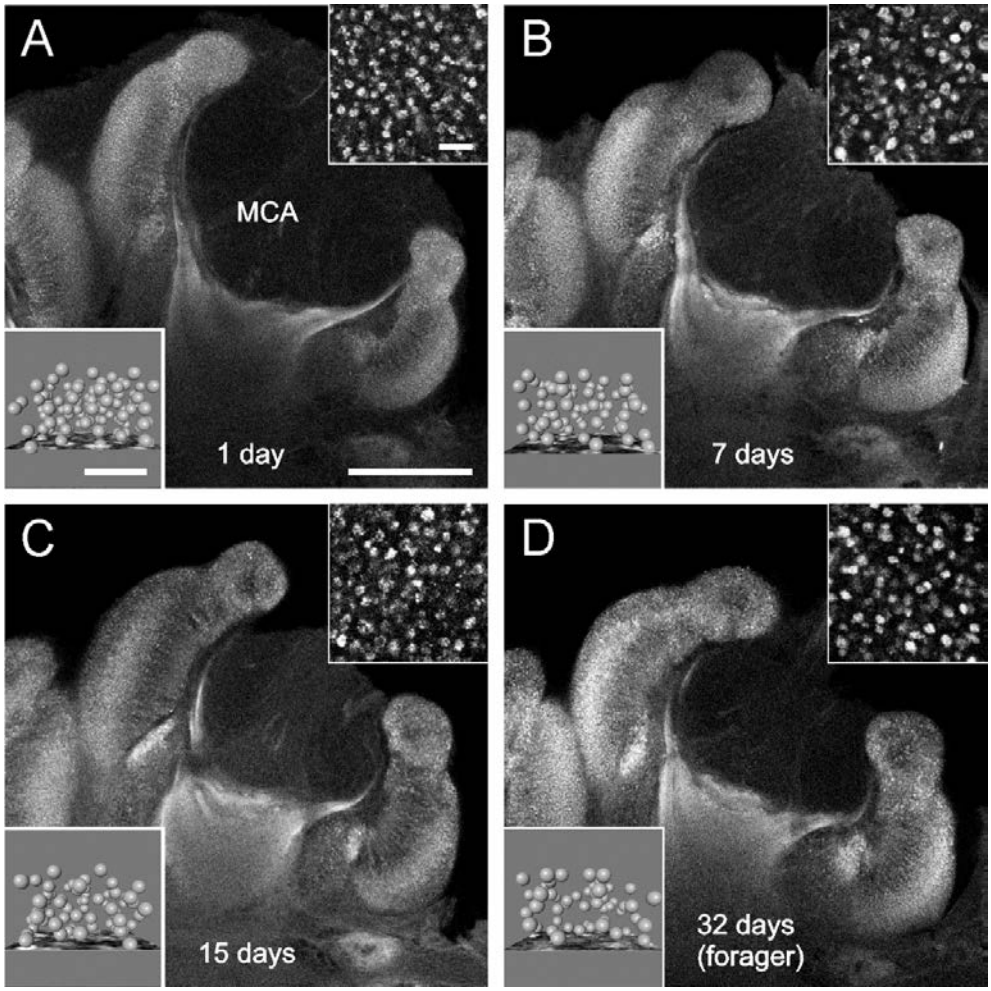


Figure 2. Age- and task-dependent plasticity in the mushroom-body calyx. **A-D:** Synapsin labeling of the left hemisphere medial calyx illustrates a consecutive age- and task-related size/volume increase of the calyx. **Upper right corner insets:** Exemplary high magnification of the corresponding dense collar region shows a continuously synaptic bouton density decrease. **Lower left corner insets:** Exemplary 3D landmark reconstruction of synaptic density in 1000 µm³ boxes within the dense collar supports the observation of a continuously synaptic bouton density decrease. MCA: medial calyx. Scale bars = 100 µm in A-D; 5 µm in lower left and upper right insets in A-D.

Lip and dense collar showed a significant volume gain between 1-day-old bees and foragers (Fig. 3: Mann-Whitney U-test, lip: 1 day – 32 days, $p < 0.001$; dense collar: 1 day – 32 days, $p < 0.001$). This significant increase was particular distinct after the first week of adult life (Fig. 3: Mann-Whitney U-test, lip: 1 day – 7 days, +27.2 %, $p < 0.001$, dense collar: 1 day – 7 dense collar: +27.6 %, $p < 0.001$), while changes after the second week of adult life (Fig. 3: Mann-Whitney U-test, lip: 7 days – 15 days, +3.9 %, $p > 0.05$; dense collar: 7 days – 15 days, 2.1 %, $p > 0.05$) and during the subsequent transition from indoor to outdoor duties (Fig. 3: Mann-Whitney U-test, lip: 15 days – 32 days, +1.2 % $p > 0.05$; dense collar: 15 days – 32 days, -1.3 %, $p > 0.05$) were comparably less pronounced (Fig. 3). The loose collar region as well as the basal ring followed this general scheme of a volume increase during adult maturation .

Table 1: Natural maturation dependent neuronal changes in mushroom-body calyx

	1 day (n = 14)	7 days (n = 13)		15 days (n = 11)		32 days (n = 15)	
Volume	**Volume ($\mu m^3 \times 10^6$)**	**Volume ($\mu m^3 \times 10^6$)**	**Change (%)**	**Volume ($\mu m^3 \times 10^6$)**	**Change (%)**	**Volume ($\mu m^3 \times 10^6$)**	**Change (%)**
Lip	4.00 ± 0.32	5.09 ± 0.26	27.2	5.30 ± 0.34	3.9	5.36 ± 0.41	1,2
Collar (dense)	3.93 ± 0.30	5.02 ± 0.30	27.6	5.13 ± 0.50	2.1	5.06 ± 0.30	-1,3
Collar (loose)	1.67 ± 0.22	2.22 ± 0.25	32.9	2.02 ± 0.28	-9.1	1.86 ± 0.19	-7,6
Basalring	1.47 ± 0.16	1.75 ± 0.12	18.9	1.76 ± 0.11	0.6	1.79 ± 0.14	1,6
Boutons	**Boutons (per 1000μm^3)**	**Boutons (per 1000μm^3)**	**Change (%)**	**Boutons (per 1000μm^3)**	**Change (%)**	**Boutons (per 1000μm^3)**	**Change (%)**
Lip	57.36 ± 2.41	46.73 ± 3.18	-18.5	39.68 ± 2.83	-15.1	35.57 ± 2.42	-10.4
Collar (dense)	94.49 ± 4.27	75.65 ± 5.32	-19.9	70.14 ± 7.13	-7.3	63.00 ± 4.24	-10.2
Overall Boutons	**Boutons per calyx ($\times 10^5$)**	**Boutons per calyx ($\times 10^5$)**	**Change (%)**	**Boutons per calyx ($\times 10^5$)**	**Change (%)**	**Boutons per calyx ($\times 10^5$)**	**Change (%)**
Lip	2.30 ± 0.20	2.38 ± 0.19	3.6	2.10 ± 0.16	-11.9	1.91 ± 0.20	-8.9
Collar (dense)	3.71 ± 0.27	3.80 ± 0.29	2.2	3.60 ± 0.42	-5.5	3.18 ± 0.21	-11.5

Taking analyses of neuronal plasticity processes in the MB calyx further from pure volume measurements, we analyzed synapsin positive bouton densities in the lip and the dense collar region. These were shown to correlate well with the densities of presynaptic projection neuron boutons (Groh et al., 2012). The two regions were chosen as they express a rather homogenous distribution of clearly identifiable synaptic boutons (Fig. 1B) (Stieb et al., 2010; Groh et al., 2012). Three-dimensional quantification of synapsin positive profiles in defined sample volumes of 1000 µm³ (3 per dense collar, ROI 1-3 Fig. 1B; four per lip, ROI 4-7 Fig. 1B) revealed a strong decrease of the synaptic density for both, the lip and dense collar region from 1-day-old bees to 32-day-old foragers (Table 1). Based on a qualitative comparison, this decrease in density becomes very obvious in the increase in distances of synaptic bouton profiles and a decrease in their packing density (insets of Fig. 2A-D – corresponding to Fig. 1B ROI 2).

Taking the volume increase into account, we asked whether synaptic boutons are just moving apart while remaining constant in number or whether the total number of synapsin positive boutons changes. For this, density measurements were extrapolated to the reconstructed volumes of the corresponding calyx subregions. During the first week of adult life total MG numbers, statistically, did not change. However, we observed a slight increase in the lip and dense collar (Fig. 3: Mann-Whitney U-test, lip: 1 day – 7 days, +3.6%, $p > 0.05$; dense collar: 1 day – 7 days, +2.2%, $p > 0.05$). This tendency is totally reverted after the second week during which total MG numbers were reduced (Fig. 3: Mann-Whitney U-test, lip: 7 days – 15 days, -11.9%, $p < 0.05$; dense collar: 7 days – 15 days, -5.5%, $p > 0.05$). With the subsequent transition from nursing to foraging the total MG numbers significantly decreased in both calyx subregions (Fig. 3: Mann-Whitney U-test, lip: 15 days – 32 days, -8.9%, $p < 0.05$; dense collar: 15 days – 32 days, -11.5%, $p < 0.05$). This shows that the net volume increase of the MB calyx is accompanied by a net decrease of presynaptic boutons in the lip and the dense collar region between 1-day-old bees and foragers (Fig. 3: Mann-Whitney U-test, lip: 1 day – 32 days, $p < 0.001$; dense collar: 1-day – 32-days, $p < 0.001$). As MG densities decreased at all analyzed intervals the extrapolations clearly show that MG densities alone can be misleading. Only in combination with volume measurements they allow conclusions regarding changes in total MG numbers.

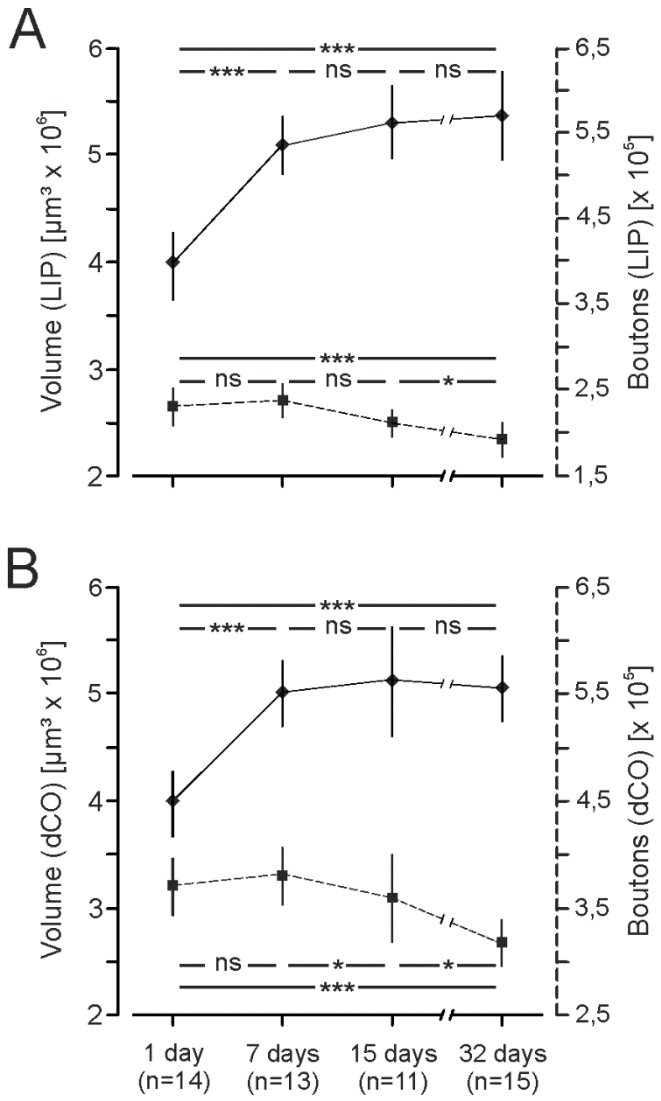


Figure 3. Age- and task-dependent neuronal plasticity in mushroom body calyx. **A:** Structural plasticity in the lip. The tremendous lip volume increase (continuous line) from 1-day-old bees to 32-day-old foragers mainly happens during the first week of adult life and is accompanied by a highly significant net decrease in synaptic boutons that is strongest during the second week of adult life and the transition to foraging (dashed line). **B:** Structural plasticity in the dense collar region. Similar to the lip, the dense collar increases in volume from 1-day-old bees to 32-day-old foragers. This mainly takes place in the first week of adult life (continuous line) and is again accompanied by a highly significant overall decrease in synaptic boutons that is strongest during the transition to foraging (dashed line).

Cellular processes underlying neuronal plasticity

The age- and task dependent volume expansion of the calyx neuropil in combination with the net decrease of MG leads to question of what actually causes the volume expansion of the calyx - what fills the expanding 'gaps' in between the presynaptic boutons? To answer these questions, we used the same age groups from our age-cohort experiments and labeled agarose sections for f-actin and β-tubulin (Fig. 4A-I). F-actin-phalloidin positive profiles

formed a thin corona around the synapsin positive boutons leaving gaps between individual MGs (Fig. 1B, C). F-actin was shown to be enriched in the dendritic tips of the KCs (Frambach et al., 2004; Pasch et al. 2011; Groh et al., 2012). This pattern remained constant over the time course of adult maturation, but appeared more distinct in older bees compared to freshly eclosed 1-day-old bees (data not shown). In contrast to f-actin-phalloidin, β-tubulin positive profiles were shown to be enriched in the main dendritic branches of KCs (Stieb et al., 2010). Qualitative inspection of our double labeling confirmed that all 'gaps' between the MGs (indicated by the f-actin labeling) were occupied by β-tubulin positive profiles and showed little to no overlap with the f-actin-phalloidin labeling (Fig. 4A-I). This general pattern persisted throughout the investigated stages of adult maturation. While MG densities decreased with age the increasing gaps between MG remained filled with β-tubulin positive profiles (representative examples are shown in Fig. 4C, F, I). Particularly in the loose collar, but also in the center of the lip, β-tubulin positive profiles formed thick bundles connected to groups of KC somata (e.g. Fig. 4A, B). Selective neuronal tracings of only a few KCs in combination with anti β-tubulin labeling showed a clear co-localization (example shown in Fig. 4J) supporting the presence of β-tubulin in main KC dendritic branches and leading to the conclusion that individual MG are pushed apart by growth of the KC dendritic network.

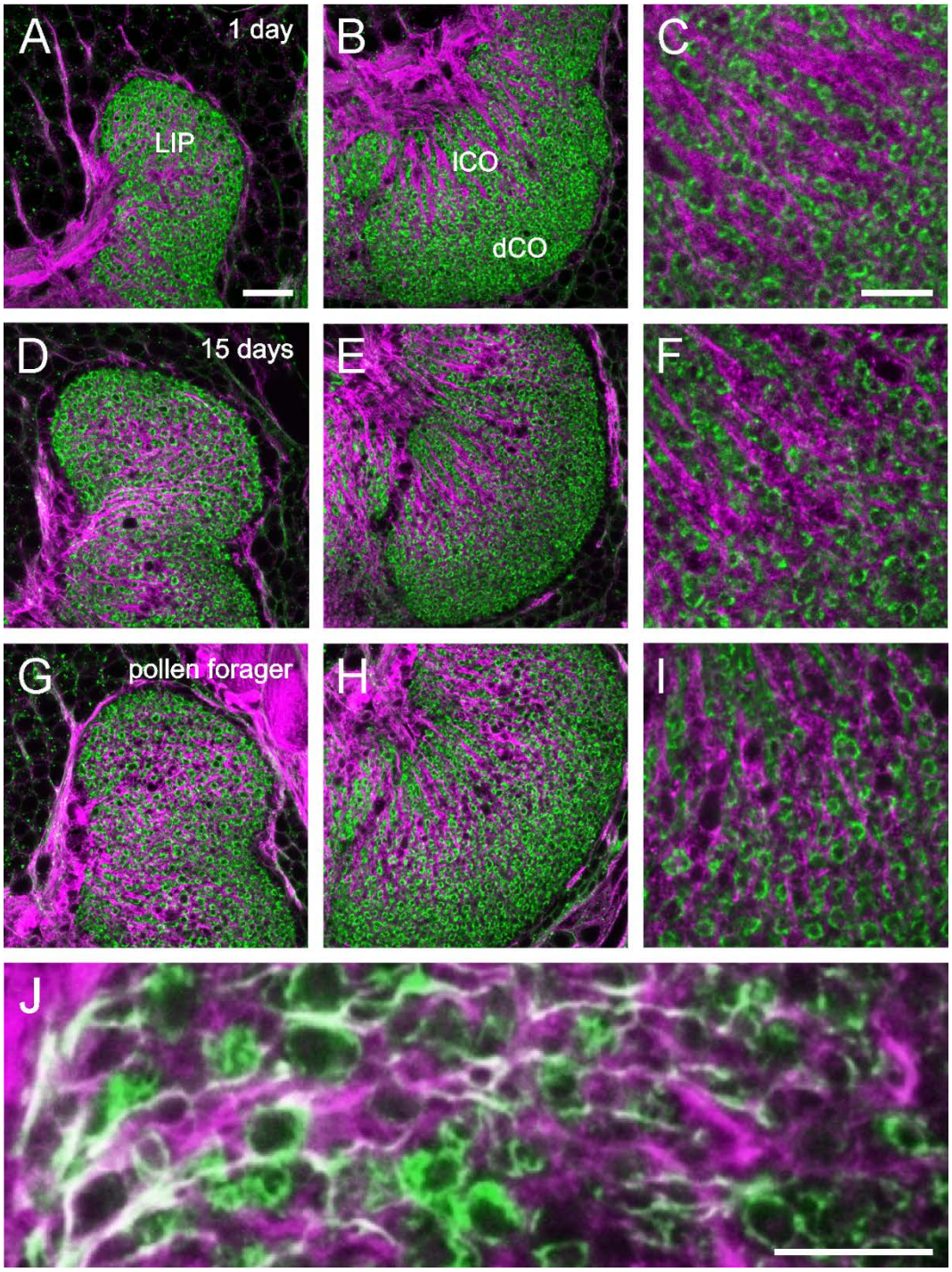


Figure 4. Possible mechanism underlying volume expansion of the mushroom body calyx. **A-I:** Different magnification of the calyx and its subregions labeled for f-actin (green) and β-tubulin (magenta) illustrate, that all gaps between individual microglomeruli (f-actin profiles) are occupied by β-tubulin rich fibers originating from the Kenyon cells. This observation is irrespective of age and task even though synaptic density decreases in the time course of adult maturation and gaps become larger. **C, F and I:** High magnifications show little to no overlap between f-actin positive microglomeruli profiles and tubulin rich Kenyon cell fibers. **J:** Neuronal tracing of few Kenyon cells (green) double labeled for β-tubulin (magenta) confirms that Kenyon cell fibers actually lie in between the microglomeruli and contain β-tubulin. dCO: dense collar, lCO: loose collar, LIP: lip. Scale bars = 20 µm in A, B, D, E, G and H; 10 µm in C, F, I and J.

Potential pheromonal modulation of synaptic maturation

To investigate potential influences of the primer pheromone EO on synaptic maturation processes, 6 pairs of triple cohort colonies were established. One colony of each pair was treated with an additional EO source, while the other colony served as a control (Leoncini et al., 2004a). In contrast to the original experiment by Leoncini et al. (2004a), artificial application of EO did not always induce a robust delay in the onset of foraging in the treated colonies compared to the control colonies (Fig. 5). A statistically significant delay of first foraging was found only in 2 out of 6 cases (Fig. 5: Mann-Whitney-Test, B: +3.8 days, $p < 0.01$; E: +3.0 days, $p < 0.05$), and one colony which showed a trend towards a delayed onset (Fig. 5C: Mann-Whitney U-test, +1.9 days, $p > 0.05$). Surprisingly, two other pairs showed a strong effect in the opposite direction with control colonies being delayed compared to the EO treated ones (Fig. 5: Mann-Whitney U-test, A: -5.7 days, $p < 0.01$; D: -2.7 days, $p < 0.05$) and one pair of colonies that did not differ at all (Fig. 5C: Mann-Whitney U-test, -0.2 days, $p > 0.05$).

The results of our natural neuronal maturation experiments (Fig. 2-4) have demonstrated that MG densities in the MB calyx significantly change with age and task and, therefore, represent a good measure for the status of adult synaptic maturation. MG densities in the EO manipulated vs. control experiments was measured in 2D in the dense collar only (similar to the method used by Stieb et al. 2010, 2012 and Scholl et al. 2014). Comparable to our 3D quantifications, we found a significant reduction in the density of synapsin positive boutons within the dense collar between 7-day-old and 15-day-old bees in 8 out of 12 colonies (Fig. 5: Mann-Whitney U-test, A(EO): -3.81 MG, $p < 0.001$; A(CON): -5.81 MG, $p < 0.001$; B(CON): -3.34 MG, $p < 0.01$; C(EO): -4.89 MG, $p < 0.001$; C(CON): -4.85 MG, p 0.001; D(EO): -4.28 MG, $p < 0.001$; D(CON): -4.30 MG, $p < 0.001$; E(CON): -2.33 MG, $p < 0.05$). In the remaining 4 colonies, we found a trend towards a density reduction (Fig. 5: Mann-Whitney U-test, B(EO): -2.59 MG, $p = 0.06$; E(EO): -2.87 MG, $p = 0.30$; F(EO): -1.45 MG, $p = 0.25$; F(CON): - 2.26 MG, $p = 0.05$). This robust observation using two alternative quantification methods (3D in whole mounts brains, 2D in agarose sectioned brains) clearly supports our finding that a decrease in synaptic density is correlated to age and task.

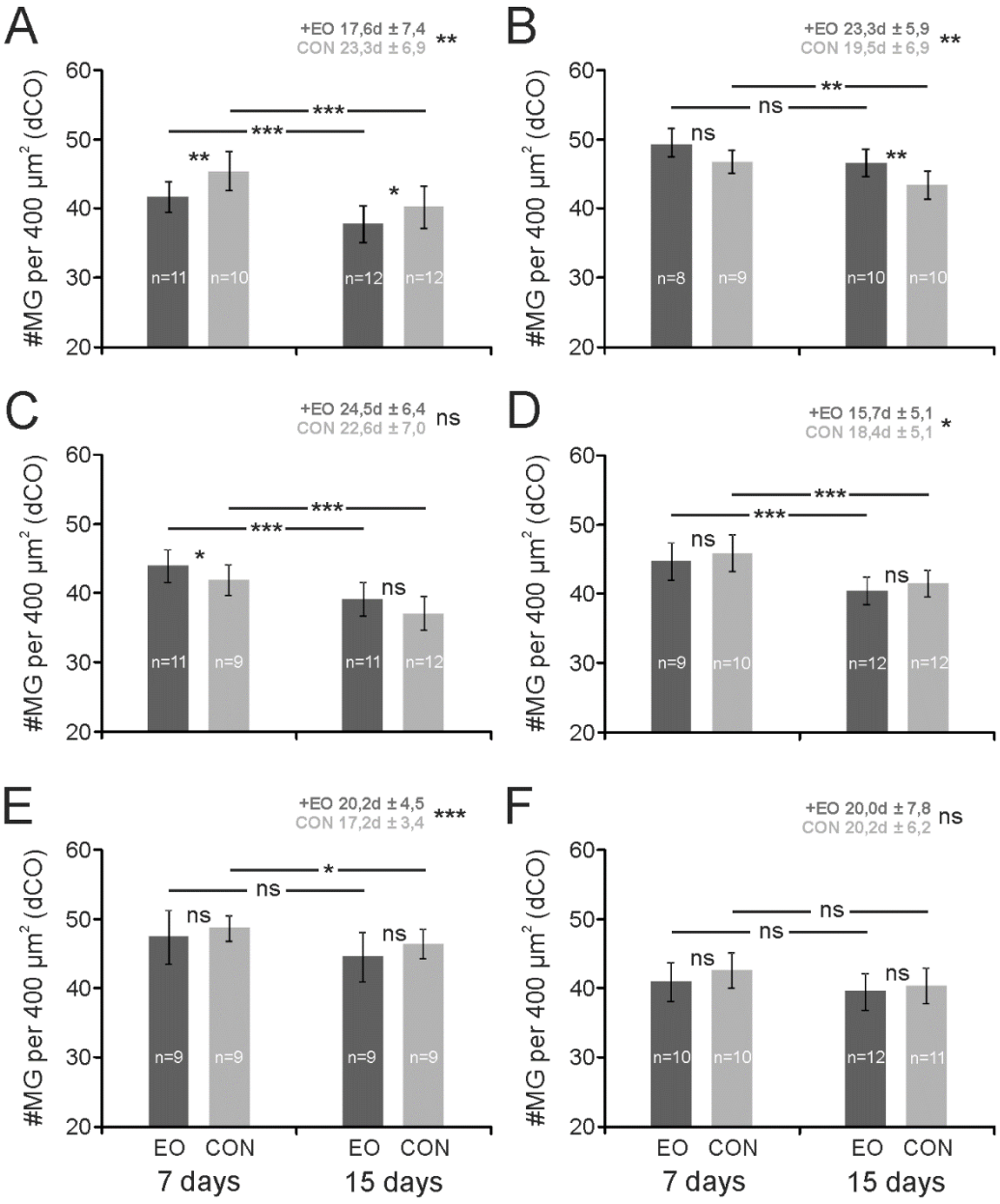


Figure 5. Ethyl oleate influence on synaptic maturation during the first two weeks of adult maturation in correlation to the onset of foraging. Confirming the results from our age-cohort (Fig. 3B and Table 1) synaptic densities in dense collar region decreased with age in 8 out of 12 triple cohort colonies significantly [A(EO), A(CON), B(CON), C(EO), C(CON), D(EO), D(CON), E(CON)] and by trend in the remaining 4 [B(EO), E(EO), F(EO), F(CON)]. Yet as only 2 out 6 paired trials (A and B) the EO treated colony showed a significantly delayed foraging onset it was not possible to correlated a synaptic maturation status to the pheromonal induced behavioral phenotype.

We hypothesized that a delayed synaptic maturation status (≙ greater MG density) during the first 2 weeks of adult maturation would predict a delayed onset of foraging in colonies treated with EO compared to the control colonies. However, as our EO manipulations did not result in a consistent behavioral response to EO as previously shown by Leoncini et al. (2004a), this hypothesis could not be tested. Even when we just took the onset of foraging into account irrespective of the treatment, only 2 out of 6 pairs (Fig. 5A, B) showed a reduced synaptic density in 15-day-old bees (Fig. 5: Mann-Whitney U-test, A: -2.42 MG/400 µm², $p < 0.05$; B: -3.18 MG/400 µm², $p < 0.01$) in the colony that actually started to forage earlier. In one of those pairs (Fig. 5A) the observed reduction in synaptic density reduction was already significantly different at day 7 of adult maturation (Fig. 5: Mann-Whitney U-test, A: -3.79 MG/400 µm², $p < 0.01$; B: -2.43 MG/400µm², $p > 0.05$). In a further pair (Fig. 5C) focal bees from one colony were reduced in synaptic density at the age of 7 days (Fig. 5C: Mann-Whitney U-test, -2.1 MG/400 µm², $p < 0.05$), revealed a non-statistically significant reduction in 15-day-old bees (Fig. 5C: Mann-Whitney U-test, -2.06 MG/400 µm², $p > 0.05$), and were by trend ahead of the other colony in the onset of foraging. In the other three remaining pairs (Fig. 5D-F) synaptic density did not differ between the treated and control colony in the 7-day-old group (Fig. 5: Mann-Whitney U-test, D: -1.13 MG/400 µm², $p > 0.05$; E: -1.32 MG/400 µm², $p > 0.05$; F: -1.69 MG/400 µm², $p > 0.05$) and the 15-day old group (Fig. 5: Mann-Whitney U-test, D: -1.11 MG/400 µm², $p > 0.05$; E: -1.86 MG/400 µm², $p > 0.05$; F: -0.88 MG/400 µm², $p > 0.05$).

Discussion

In this study we show that the MB calyx volume increase during the first week of adult life is mainly driven by an outgrowth of the KC dendritic network as a function of neonatal development. With ongoing maturation and changes in sensory environment of the bees this process is followed by a significant pruning of projection neuron axonal boutons in the lip and dense collar region of the calyx. Using the primer pheromone EO to manipulate behavioral maturation did not deliver a consistent neuronal correlate but also confirmed that MG density in the dense collar region of the MB calyx decrease with age and task.

Volume changes of the mushroom body calyx during adult maturation

The basis of division of labor within the female honeybee worker caste is a temporal polyethism that involves significant changes in the sensory environment of the bees. This has been shown to be associated with plastic changes in the MB, a higher sensory integration center in the insect brain. In line with earlier studies (Withers et al., 1993, 1995; Durst et al., 1994; Farris et al., 2001; Groh et al., 2012), our results show that adult behavioral maturation in honeybees goes along with a continuous volume increase of the MB calyx. Similar correlations of behavioral and structural neuronal plasticity have been described in other social Hymenoptera like the carpenter ant *Camponotus floridanus* (Gronenberg et al., 1996), the desert ant *Cataglyphis bicolour* (Kühn-Bühlmann and Wehner, 2006), and the paper wasp *Polybia aequatorialis* (O'Donnell et al., 2004), which all express an age- and experience induced plasticity of the MB volume. The level of detail of our consecutive age cohort, however, revealed that the calyx volume increase is strongest within the first week of adult maturation. During this time bees are normally involved in nursing duties, and sensory stimuli, in general, are restricted to the dark hive environment (Lindauer, 1952) – yet, short orientation flights may occur (Capaldi et al., 2000). This result supports the idea that the initial volume increase during the first week of adult life is at least partly an experience-independent internal program (Farris et al., 2001; Ismail et al., 2006) as it also occurs in social isolation, sensory deprivation (Fahrbach et al., 1998) and in bees that have been prevented from flying out (Withers et al., 1995). In contrast to this, no initial MB volume expansions have been observed in the solitary bee *Osmia lignaria*, probably indicating a more completed neuronal development upon adult eclosion (Withers et al., 2008), which may be assigned to the solitary lifestyle of this bee. Fahrbach et al. (1998) have defined this early volume expansion in the calyx as "experience-expectant", in contrast to "experience-dependent" volume changes which have been shown to be associated with the accumulation of foraging experience (Farris et al., 2001; Ismail et al., 2006). In our study the experience-driven volume expansion is not very pronounced, which could reflect the amount of foraging trips which have not been monitored in detail. Interestingly, it seems that the volume of the collar (loose + dense region) slightly decreases during the transition of nurse bees to foragers (Tab. 1). Most importantly, however, our results show that this period is accompanied by pruning of MG.

Neuronal plasticity mechanisms in the mushroom body calyx

In contrast to most of the preceding studies, our experimental combination of total volume and 3D MG density measurements within in the MB calyx of the same brain enabled us to analyze in great detail cellular modifications underlying the described volume changes. Consistent with Groh et al. (2012) we were able to confirm and extend the observation that the drastic MB volume increase during adult maturation is accompanied by a continuous decrease of MG in the lip as well as in the dense collar region. This raises the question whether total MG numbers remain constant. During the first week of adult life our extrapolations indicate even a slight increase in the total number of MG, which could be explained by the formation of new presynaptic boutons of still sprouting projection neurons. As this process takes place in the olfactory innervated lip as well as in the visually innervated collar during a time when the bees mostly spend their time within the dark hive, this suggests a prolonged initial neuronal development during the first week of adult life. With the second week of adult life, the total number of MG starts to decrease in both calyx subregions ending up with a significant net reduction of MG in the course of adult maturation. Such a pruning of projection neuron axonal boutons has already been suggested using density measurements in *Cataglyphis* ants (Seid and Wehner, 2009; Stieb et al., 2010) and in the honeybee (Scholl et al., 2014) and was shown to be triggered by sensory (light) exposure in the MB calyx collar. Interestingly in both cases, for *Cataglyphis* ants (Stieb et al., 2010, 2012) as well as the honeybee (Scholl et al., 2014), it was possible to induce a decrease in the MG density of dense collar region already during the first week of adult maturation, a phase during which we likewise observe a density decrease. On the other hand, we have now identified that the total number of MG actually seem to increase during this phase. It therefore remains to be shown whether the main effect of sensory stimulation is expressed in KC dendritic growth and/or synaptic pruning. Nonetheless, sensory-exposure induced neuronal plasticity, as measured at the level of MG density in the MB calyx may be a key mechanism to explain how bees as young as 7 days of age (Rösch, 1930) can become precocious foragers.

It was shown that stable long-term memory foundation leads to the formation of new MG in the MB calyx lip (Hourcade et al., 2010). However, our results show that the overall MG number, in general, is reduced during adult maturation. This excludes new projection neuron boutons as a major contributor to the volume expansion of the calyx. Yet as the individual

projection neuron bouton volume increases from young individuals to foragers in bees (Krofczik et al., 2008; Groh et al., 2012) and in the ant Pheidole dentata (Seid et al., 2005) they still may, just to a small extent, contribute to the MB volume increase. In general, however, the extensive KC dendritic network seems to be the most important factor underlying volumetric changes. Early work indicated that dendritic spines of KC express plastic changes correlated to age (Coss et al., 1980) as well as sensory exposure (Brandon and Coss, 1982). Our β-tubulin labeling, which completely overlaps with backfilled KC dendrites, to a very large extent fills the 'gaps' in between MG during all maturation stages. As similar results have been obtained from β-tubulin labelling in *Cataglyphis fortis* (Stieb et al., 2010), we conclude that KC dendritic outgrowth and branching is responsible for most of the observed volume increase in the MB calyx. This hypothesis has already been suggested for bees by Farris et al. (2001) based on Golgi impregnations showing a positive correlation of dendritic branching pattern increase in the collar region with age and foraging experience. Similar results have been obtained for the paper wasp *Polybia aequatorialis* (Jones et al., 2009). At the ultrastructural level Groh et al. (2012) were able to show an increase in the number of postsynaptic profiles contacting individual MGs by about 33% after the transition from nurse bees to foragers. Even though it is unknown whether those newly established postsynaptic profiles derive from new KC dendritic branches (established or new contacting KC) or extrinsic neurons, it further underlines that massive structural reorganization occurs in the dendritic and synaptic network.

The molecular mechanisms mediating such plastic changes in the KC dendritic network still remain largely unknown. For experience-dependent neuronal plasticity in the MB calyx, stimulation of muscarinic–type cholinergic receptors was shown to mimic foraging-related sensory exposure indicating that it is an associated and necessary factor (Ismail et al., 2006; Dobrin et al., 2011). Those plastic changes were suggested to be initiated by Rho GTPases, small monomeric G proteins, involved in the reorganization of the neuronal cytoskeleton (Dobrin and Fahrbach, 2012). A further candidate for mediating MG plasticity is calcium calmodulin depenent protein kinase II (CaMKII), which is highly localized in a major subpopulation of KCs (Kamikouchi et al., 2000; Pasch et al., 2011). This 'plasticity' mediating protein is well known for its role in learning processes and long-term memory formation (for review see Lisman et al., 2002; Mayford, 2007) in vertebrates (Malenka et al., 1989) as well as invertebrates (Lent et al., 2007) e.g. the honeybee (Matsumoto et al., 2014). As CaMKII is

highly concentrated in KC dendrites and dendritic spines, it may be mostly involved in postsynaptic (dendritic) plasticity, but long-term memory effects on presynaptic boutons (Hourcade et al., 2010) might be triggered via an unknown retrograde factor.

Pheromonal modulation of synaptic plasticity

It was shown that the MB calyx undergoes neuronal plasticity associated with age and experience. However, it is still an unanswered question whether and how external triggers, like the presence of foragers - that has been shown to alter behavioral maturation - can modulate the timing of behavioral and synaptic maturation. The concentration of the primer pheromone EO in the colony is an indicator of the presence of foragers, and it was shown that EO levels influence the onset of foraging (Leoncini et al., 2004a; b). In the present study we used this information to manipulate the behavioral ontogeny while searching for neuronal correlates - in this case MG density changes in the MB calyx. We carefully controlled for many factors that have been shown to influence adult maturation ranging from the genetic background to social factors like colony demography and pheromonal regulators such as QMP and BP concentrations. However, even with careful control of these factors, it was not possible to consistently reproduce a delayed onset of foraging in colonies that received an additional source of EO. We therefore speculate, that EO may be so deeply embedded in a concert of factors that even slight environmental changes may interfere or even counteract the effect of additional EO between treated and control colonies. For example the endogenous presence of EO in our paired assay may have varied as foragers from one colony could have foraged on more ethanol-containing nectar (from which EO is biosynthesized) resulting in higher EO concentrations (Castillo et al., 2012a). In fact seasonal as well as yearly changes of the ethanol concentration in the nectar sources occur and might lead to varying EO concentrations on foragers (Castillo et al., 2012b). This could also be an explanation why it was possible to induce a delayed onset of foraging in the treated colonies during the summer season of 2008 (Fig. 5B, E), while the summer season of 2009 (Fig. 5A, C, D, F), a very hot summer, potentially resulting in more fermented nectar, delivered quite unexpected behavioral results. In addition, administration of additional EO with sugar candy as introduced by Leoncini et al. (2004a) may not be optimal, as this treatment was based on the assumption that EO is passed between individuals via trophallaxis. More recent studies however have identified that evaporation via the cuticle and short range chemoreception is

the more likely transmission pathway for this pheromone (Castillo et al., 2012a; b; Muenz et al., 2012).

Due to these difficulties in inducing a clear behavioral phenotype it was not possible to correlate a delayed onset of foraging phenotype to a delayed synaptic maturation status as we had hypothesized. In general it seems quite likely that the observed independence of MB maturation from EO titers, may be explained with the MB calyx maturation being upstream of EO signaling in the maturation of nurse bees to foragers.

Nonetheless, the 2D MG density measurements of this experimental setup showed a reduced MG density in 15 day old bees when compared with 7 day old bees, which clearly confirms our advanced 3D MG density results. This underlines that MG density can be used as an adequate measure of MB calyx maturation. Future studies will have to address the fundamental question whether pheromones that influence adult maturation such as QMP (Pankiw et al., 1998a; Le Conte and Hefetz, 2008) directly trigger physiological changes in the brain via the hormonal system in e.g. juvenile hormone (Fahrbach and Robinson, 1996; Fahrbach et al., 2003) or by altering behavioral patterns that in e.g. would lead to differences in activity levels and sensory exposure, which ultimately cause neuronal and synaptic plasticity in the MB calyx (Stieb et al., 2010, 2012; Scholl et al., 2014). In this regard EO would be a very interesting pheromone with respect to possible releaser effects, but also because it has been shown that EO production is increased by the juvenile hormone analog methoprene (Castillo et al., 2012b). The well described primer pheromone QMP may serve as a reference point for future directions, as it has been demonstrated to cause releaser as well as primer effects (Slessor et al., 2005). It has been shown to directly influence juvenile hormone synthesis in recipient bees (Kaatz et al., 1992). Furthermore, a component of QMP, homovanillyl alcohol, structurally resembles dopamine in turn modulating brain dopamine function (Beggs et al., 2007; Beggs and Mercer, 2009) and behavioral patterns (Vergoz et al., 2007). It is not unlikely that pheromonal effects of EO are mediated in comparable ways.

Conclusions

For pupal development it has been shown that varying environmental factors like brood rearing temperature have a large impact on the synaptic architecture in the MB calyx (Groh et al., 2004, 2006). However, it seems that honey bees similar to *Cataglyphis* ants (Seid and Wehner, 2009; Stieb et al., 2010, 2012) also undergo an extended neonatal development

during approximately the first week after eclosion providing an experience-expectant plasticity in the MB calyx. We speculate that this prolonged neuronal development could finally bring the neuropil to a status allowing for advanced levels of experience-dependent plasticity.

However, taken the fact that precocious foragers can be induced in addition to the observation that sensory manipulations during the first week of adulthood lead to advanced synaptic plasticity shows that neuronal development can be influenced at this early phase. From this we conclude that there are at least four different, partly counteracting, mechanisms involved in the neuronal plasticity of the MB calyx: 1) prolonged initial axonal sprouting of MB extrinsic neurons during the first week of adult life, 2) age- and experience-dependent outgrowth of the KC dendritic network, 3) age- and experience-driven pruning of projection neuron boutons, and 4) learning and memory associated formation of new projection neuron boutons.

The question how internal and external factors interact to influence adult neuronal maturation in ways that translate into behavioral changes remains a chicken-egg problem still to be solved. Future studies will have to carefully disentangle the interplay of pheromones, sensory stimuli, internal programs and the hormonal system to better understand the timing of behavioral transitions and the underlying effects at the level of the brain.

Author contributions

TSM: experimental planning and discussion, conduction of neuroanatomical and behavior experiments, data analysis, writing of the manuscript. CG: experimental planning, participation in neuroanatomical and behavior experiments, data analysis. AM: experimental planning and execution of behavior experiments in Avignon in 2008 and 2009. YL: experimental planning and discussion, provided apiary space and bees in Avignon in 2008 and 2009. EP: experimental planning and discussion, principal investigator (PI) of the HFSP grant. WR: experimental planning and discussion, supervision of all neuroanatomical experiments, writing of the manuscript.

Acknowledgments

We appreciate the help of Kornelia Grübel for histology and immunostaining assistance, Thomas LeConte for setting up the pheromone manipulation experiments, Dirk Ahrens for his beekeeping expertise, and Katrin Gehring for assistance with tracing experiments. Funding was provided by HFSP (RGP0042/2007) to EP(PI), YLC and WR.

Chapter II

CaMKII is differentially localized in synaptic regions of Kenyon cells within the mushroom bodies of the honeybee brain

Abstract

Calcium/calmodulin-dependent protein kinase II (CaMKII) has been linked to neuronal plasticity associated with long-term potentiation as well as structural synaptic plasticity. Previous work in adult honeybees has shown that a single CaMKII gene is strongly expressed in the mushroom bodies (MBs), brain centers associated with sensory integration, and learning and memory formation. To study a potential role of CaMKII in synaptic plasticity, the cellular and subcellular distribution of activated (phosphorylated) pCaMKII protein was investigated at various life stages of the honeybee using immunocytochemistry, confocal microscopy, and western blot analyses. Whereas at pupal stages 3–4 most parts of the brain showed high levels of pCaMKII immunoreactivity, the protein was predominantly concentrated in the MBs in the adult brain. The results show that pCaMKII is present in a specific subpopulation of Kenyon cells, the noncompact cells. Within the olfactory (lip) and visual (collar) subregion of the MB calyx neuropil pCaMKII was colocalized with f-actin in postsynaptic compartments of microglomeruli, indicating that it is enriched in Kenyon cell dendritic spines. This suggests a potential role of CaMKII in Kenyon cell dendritic plasticity. Interestingly, pCaMKII protein was absent in two other types of Kenyon cells, the inner compact cells associated with the multimodal basal ring and the outer compact cells. During adult behavioral maturation from nurse bees to foragers, pCaMKII distribution remained essentially similar at the qualitative level, suggesting a potential role in dendritic plasticity of Kenyon cells throughout the entire life span of a worker bee.

With kind permission from John Wiley and Sons Ltd.:

Pasch E*, Muenz TS*, Rössler W. 2011. CaMKII is differentially localized in synaptic regions of Kenyon cells within the mushroom bodies of the honeybee brain. Journal of Comparative Neurology 519:3700–3712. *equally contributing first authors

The original article is available at:

http://onlinelibrary.wiley.com/doi/10.1002/cne.22683/abstract

Introduction

Social behavior in honeybees includes a remarkable range of capabilities like multimodal communication, spatiotemporal orientation, as well as learning and memory (for a recent review, see Menzel and Giurfa, 2006). After emergence from brood cells, honeybee workers spend the first 3 weeks inside the hive, except for some short orientation flights toward the end of this period before they become foragers (Lindauer, 1961). The transition from indoor to outdoor duties requires the activation of a completely different behavioral repertoire including major changes in sensory input, in particular visual and olfactory stimuli, and associated novel learning and memory tasks. Due to these remarkable changes in behavior the honeybee represents an ideal model system for the study of the neuronal mechanisms underlying behavioral plasticity.

The mushroom bodies (MBs), prominent centers in the honeybee brain, have been associated with higher sensory integration, spatial orientation, as well as learning and memory (Erber et al., 1980; Heisenberg, 1998; Strausfeld et al., 1998; Menzel, 1999, 2001; Zars, 2000; Heisenberg, 2003; Gerber et al., 2004; Menzel and Giurfa, 2006). Furthermore, previous work has shown that the transition from nurse bees to foragers is correlated with a volume increase in the MBs (Withers et al., 1993; Durst et al., 1994; Fahrbach et al., 1998; Ismail et al., 2006) and associated increases in dendritic branching of MB intrinsic neurons, the Kenyon cells (KCs), in visual regions of the MB calyx (Farris et al., 2001). A recent study in the desert ant *Cataglyphis fortis* has shown that the transition to foraging is associated with KC dendritic growth and, at the same time, pruning of synaptic complexes (microglomeruli, MG) in the visual MB calyx (Stieb et al., 2010). Both effects could be triggered by light exposure, suggesting that these processes are driven by sensory activity rather than an internal program. In the same line a recent study in *Drosophila* demonstrates that activity in olfactory projection neurons drives structural changes in MB calyx MG (Kremer et al., 2010).

Individual MG comprise large presynaptic boutons from projection neurons (PNs) forming synaptic contacts with numerous KC dendritic spines (Ganeshina and Menzel, 2001; Yasuyama et al., 2002; Frambach et al., 2004; Leiss et al., 2009). The adult number and density of MB calyx MG was shown to be affected by brood care, sensory environment, and age (Groh et al., 2004, 2006; Groh and Rössler, 2008; Krofczik et al., 2008; Stieb et al., 2010), while the formation of protein-synthesis dependent stable olfactory long term memory

(LTM) was shown to lead to an increase in the number of olfactory MG (Hourcade et al., 2010). Taken together, there is mounting evidence for a high degree of structural synaptic plasticity in MB calyx MG, but the molecular pathways mediating developmental and adult structural plasticity in these synaptic microcircuits remain elusive.

The learning-associated protein calcium/calmodulin-dependent kinase II (CaMKII) was found in the nervous system of vertebrates and invertebrates (Lisman and Goldring, 1988; Lisman, 1989; Silva et al., 1992a, b; Soderling, 1993; Kamikouchi et al., 2000). In rat hippocampus CaMKII contributes up to 2% of the total protein mass (Erondu and Kennedy, 1985) and is thought to be a primary initiating signal in the memory consolidation pathway (Mayford, 2007). CaMKII in dendritic spines is activated by an increase of intracellular Ca^{2+} and can rapidly be autophosphorylated, leading to a persistently active form (Lou et al., 1986; Miller and Kennedy, 1986; Schworer et al., 1986; Barria et al., 1997; Kelleher et al., 2004; Rosenberg et al., 2005). Whether continuous activation of CaMKII is required for long-term potentiation (LTP) maintenance and memory formation is controversial (Chen et al., 2001; Kelleher et al., 2004; Irvine et al., 2005). Besides its key role in the induction of LTP (reviewed in Lisman et al., 2002), CaMKII was shown to be a major component of the postsynaptic density (Kennedy et al., 1983) and plays a role in processes mediating long-term plasticity, partly by direct interaction with cytoskeletal proteins inducing changes in spine size and shape (Mayford, 2007; Abraham, 2008; Okamoto et al., 2009).

Several studies have also suggested a role of CaMKII in neuronal plasticity in insects. For example, in the moth Manduca sexta, CaMKII is likely involved in large-scale dendritic remodeling during postembryonic development (Burkert and Duch, 2006). In the honeybee Ca^{2+} was shown to be necessary and sufficient for the formation of protein-synthesis-dependent LTM, suggesting a potential role of CaMKII in mediating plasticity (Perisse et al., 2009). Expression studies of the single CaMKII gene in *Apis mellifera* revealed a differential distribution in MB neurons with particularly high concentrations in cell bodies of the noncompact KCs (Kamikouchi et al., 2000). Multimodal learning experiments in the cockroach led to changes in pCaMKII protein levels in the MBs, further supporting a potential role of CaMKII in plasticity processes in the insect brain (Lent et al., 2007).

In the present study we mapped activated (phosphorylated) CaMKII protein during different life stages in the honeybee brain. Given a potential role of CaMKII in mediating structural

synaptic plasticity associated with behavioral transitions and LTM, the precise localization in synaptic compartments is of paramount importance. We examined the cellular distribution and specificity of Thr286 phosphorylated CaMKII protein by western blotting, immunolabeling, and confocal analyses. Furthermore, developmental, age-, and task-dependent changes of pCaMKII were investigated in age cohorts.

Material and Methods

Animals and cohort experiments

For all experiments, worker honeybees (*Apis mellifera carnica*) from the apiary at the Biozentrum, University of Würzburg, Germany were used. A cohort experiment was carried out to obtain age-controlled bees. To synchronize brood, an egg-laying queen was confined to a single brood comb for 24 hours by using a wire-mesh cage that permitted passage of workers only. A few days before emergence the comb was transferred into an incubator (Memmert IPP 500, Schwabach, Germany), and kept at high humidity (60–70%) with a constant temperature of 35°C, close to the conditions in pupal cells (Seeley, 1985). After emergence 1-day-old (≤24 hours) honeybees (≈400) were collected from the comb, labeled with a white color spot on the thorax, and transferred back into the colony. Marked bees were collected at ages of 1 (n = 10) and 8 (n = 10) day(s) from the comb, while the group of 29-day-old bees (n = 10) were nectar foragers caught at an artificial feeder. Bees at pupal stages 3 (n = 4) and late 4 (n = 6) (P3 and P4) were identified according to previously established criteria (Eichmüller, 1994; Groh and Rössler, 2008).

Immunocytochemistry

For immunolabeling, bees were anesthetized with CO2, decapitated, and the heads were placed 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). After dissection, brains were transferred into cold 4% formaldehyde in phosphate-buffered saline (PBS, pH 7.2) overnight at 4°C. Fixed brains were rinsed in PBS three times, 10 minutes each step, embedded in a frontal position in 5% low-melting-point agarose (Agarose type II, No. 210-815, Amresco, Solon, OH) and sectioned at 100 µm thickness with a vibrating microtome (Leica VT 1000S, Nussloch, Germany). Free-floating agarose sections were rinsed one time in PBS with 2%

Triton X-100 (PBST), two times in 0.2% PBST (10min each), and then preincubated in 0.2% PBST and 2% normal goat serum (NGS; ICN Biomedicals, no. 191356, Orsay, France) for 1 hour at room temperature. To label neuronal f-actin, sections were incubated in 0.2 units of Alexa Fluor 568 phalloidin (Molecular Probes, A-12380, Leiden, The Netherlands) in 500 µl 0.2% PBST with 2% NGS for three nights at 4°C. Sections were simultaneously incubated with a polyclonal antibody to the phosphorylated calcium/calmodulin-dependent protein kinase II α (pCaMKIIα; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, sc-12886-R). For localization experiments, incubation of anti-pCaMKIIα antibody (1:500) in 500 µl 0.2% PBST and 2% NGS was combined with a monoclonal antibody to the *Drosophila* synaptic-vesicle-associated protein synapsin I (1:10; SYNORF1; kindly provided by E. Buchner, University of Würzburg, Germany) (Klagges et al., 1996). Details about the specificity of these two antibodies in honeybee brain tissue are listed in Table 1 and the antibody characterization section. Alternatively, incubation was combined with 0.2 units of Alexa Fluor 568 phalloidin. Afterwards, preparations were rinsed in five changes of PBS and incubated in Alexa 488-conjugated goat antirabbit secondary antibody (1:250; Molecular Probes, A-11008) in PBS with 1% NGS for 2 hours at room temperature or Alexa 568-conjugated goat antimouse secondary antibody (1:250; Molecular Probes, A-11004). To label cell nuclei, sections were subsequently incubated for 15 minutes in Hoechst 34580 (1:500; Molecular Probes, H-21486) in PBS at room temperature. Finally, sections were rinsed in at least five changes of PBS, transferred into 60% glycerol/PBS for 30 minutes, and mounted in 80% glycerol/PBS on slides.

Primary antibody characterization

pCaMKII antibody is a polyclonal affinity-purified rabbit antibody raised against a short amino acid sequence containing phosphorylated Thr286 of CaMKIIα of human origin. The exact epitope sequence (aa 282 HRQET(-phospho)VDCLK 291) was determined by Kojundzic et al. (2010) by analyzing the blocking peptide using mass spectrometry. The epitope sequence is highly conserved in the honeybee genome as revealed by blast analysis (Honeybee Genome Sequencing Consortium, 2006). In western blot analyses of honeybee brain homogenate the antibody recognized four prominent bands between 50–65 kDa ranging around the expected molecular weight of ≈50 kDa for CaMKII (Santa Cruz Biotechnology, datasheet). Specificity was further confirmed by preabsorption control

experiments using the appropriate blocking peptide (Santa Cruz Biotechnology, sc-12886 P) which delimited all bands (Fig. 1).

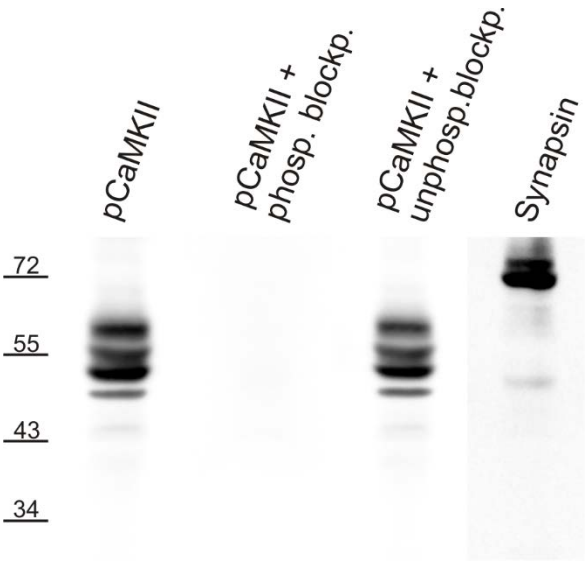


Figure 1. Immunoblots of pCaMKII and synapsin. Anti-pCaMKII antibody recognizes multiple bands between ≈50 and ≈65 kDa (left lane). The signal is completely quenched when pCaMKII antibody was preincubated with the phosphorylated blocking peptide (first middle lane). No change in signal strength was visible if pCaMKII was preincubated with the unphosphorylated blocking peptide (second middle lane) in comparison with pCaMKII alone. Synapsin antibody (SYNORF1) recognizes two prominent bands at ≈72 kDa (right lane). Molecular weights are indicated in kDa.

The synapsin antibody is a monoclonal mouse antibody raised against the *Drosophila* melanogaster synapsin Glutheion-S-transferase fusion protein which has been created in E. coli (Klagges et al., 1996). Western blot analyses of *Drosophila* brain homogenate revealed three prominent bands at 70, 74, and 80 kDa and a doublet at 143 kDa. The exact recognized epitope (aa 341 LFGGMEVCGL 350) has recently been described by Hofbauer et al. (2009). This antibody was previously shown to label presynaptic terminals in similar patterns in the honeybee (Kirschner et al., 2006) and across a wide range of neopteran insects (Groh and Rössler, 2011). In honeybee brain homogenates two prominent bands at ≈72 kDa were detected by the antibody (Fig. 1).

Table 1. Antibodies used

Antigen	Immunogen	Manufacturer	Dilution used
pCaMKIIα	Aa 282 HRQET(-phospho)VDCLK 291 containing phosphorylated Thr-286 of CaMKII of human origin, protein accession #Q9UQM7*	Rabbit polyclonal, Santa Cruz Biotechnology; sc-12886-R	1:500
Synapsin	*Drosophila* synapsin GST fusion protein, protein accession #CAA64723	Mouse monoclonal, Klagges et al. (1996); Developmental Studies Hybridoma Bank; 3C11 (anti SYNORF1)	1:10

* Using mass spectrometry to analyse the blocking peptide Kojundzic et al. (2009) revealed the exact epitope of 10 amino acids (282-291) the antibody was raised against.

Confocal laser scanning microscopy and image processing

Preparations were visualized with a confocal laser scanning microscope (Leica TCS SP2, Leica Microsystems, Wetzlar, Germany) equipped with an argon/krypton laser and three diode lasers. For comparison of certain brain regions (in particular the MB calyces) between different individuals, all images were taken at a defined plane in the central brain. In this plane the MB calyces and a set of landmarks were visible: the upper and lower division of the central body, and the pedunculi of the MBs. Within this plane the innermost calyx branches of the medial calyces were scanned at a high resolution (HCX PL APO Ibd.BL 63× 1.4 OIL, digital zoom 1 and for a more detailed view zoom 1.5, 2, or 4), 5–10 µm underneath the surface of the sections to avoid artifacts caused by penetration differences of the anti-pCaMKII antibody. Scans of the vertical lobe were produced accordingly. In double- or triple-labeled preparations the two or three channels were merged with the use of pseudocolors. Images were processed with FIJI (ImageJ 1.44c; Wayne Rasband, National Institutes of Health, Bethesda, MD) and Corel Draw X3 software (Corel, Ottawa, ON, Canada) and, in some cases, adjusted for brightness and contrast using the same settings for comparative data.

Immunoblotting

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 4% stacking and 10% separating gel. Worker bees were collected from a hive and chilled on ice for several minutes until they stopped moving. The bees were mounted in small metal tubes with their head free on the upper side. While continuously cooling the bees, their brains were dissected and immediately transferred into 70 µl Laemmli buffer before being snap-frozen in liquid nitrogen. The brains were homogenated on ice, centrifuged, and the supernatant heated to 95°C for 5 minutes. The homogenate (two brains) was subjected to SDS-PAGE and blotted on a nitrocellulose membrane. To determine molecular weights a prestained SDS marker (Fermentas, SM0671) was used. After blocking the membrane against nonspecific binding in 5% bovine serum albumin (BSA) dissolved in 0.1% TBST the membrane was incubated with the anti-pCaMKII primary antibody (1:4,000; Santa Cruz Biotechnology, sc-12886-R) or synapsin primary antibody (1:100, SYNORF1; kindly provided by E. Buchner, University of Würzburg, Germany) in TBST with 5% BSA overnight at 4°C. For preabsorption experiments anti-pCaMKII antibody was first diluted 1:1,000 in TBST with 5% BSA and a 5-

fold (by weight; 1:200) excess of the phosphorylated blocking peptide (Santa Cruz Biotechnology, sc-12886 P) or of the blocking peptide without a phosphorylated Thr (Santa Cruz Biotechnology, con-12886 P) was added. Both blocking peptides were matched with the pCaMKII antibody. After incubation for 2 hours at room temperature the mixtures were diluted 1:3 in TBST with 5% BSA and the membranes were incubated overnight at 4°C. The membranes were then rinsed in three changes of TBST and incubated in horseradish peroxidase (HRP)-conjugated goat antirabbit IgG secondary antibody (1:10,000; Biorad, 170-6515) or antimouse IgG secondary antibody (1:3,000; Thermo Scientific, Waltham, MA, 31444) in TBST with 5% BSA for 1 hour at room temperature. After rinsing the membranes in three changes of TBST, the SuperSignal West Pico Substrate, ECL Kit (Thermo Scientific, 34080) was used to visualize immunoreactivity.

Results

Immunoblotting

To test whether the antibody specifically recognizes the pCaMKII protein, immunoblots using honeybee brain tissue were performed. The antibody recognized multiple bands (four very prominent bands and a very weak fifth band at a lower molecular weight, which might represent a degradation product) in the honeybee brain homogenate corresponding to molecular weights between ≈50 to ≈65 kDa (Fig. 1, left lane; pCaMKII). This is in a similar range compared to results in *Drosophila* (Takamatsu et al., 2003). When the antibody was preincubated with an excess of the blocking peptide containing pThr286, immunoreactivity (IR) was completely quenched (Fig. 1, first middle lane; pCaMKII + phos. blockp.). In contrast, IR was unaffected when the antibody was preincubated with an excess of the unphosphorylated blocking peptide (Fig. 1, second middle lane, pCaMKII + unphos. blockp.), indicating that the antibody specifically recognized the phosphorylated CaMKII protein. The D. melanogaster originated antibody SYNORF1 showed strong crossreactivity with A. mellifera brain homogenate and generated two prominent bands at ≈72 kDa (Fig. 1, right lane; synapsin).

General distribution of pCaMKII in the brain

To visualize the distribution and localization of pCaMKII within different neuropils in the honeybee brain, a combination of various labeling techniques was used. To reveal information about the general distribution of the protein in major neuropil regions, we examined the fluorescent staining of pCaMKII IR throughout the brain at pupal-stage 3 (P3), late pupal-stage 4 (P4), as well as in adult brains. As the staining patterns were similar in different preparations, all figures show representative examples. The numbers of samples are given in Materials and Methods.

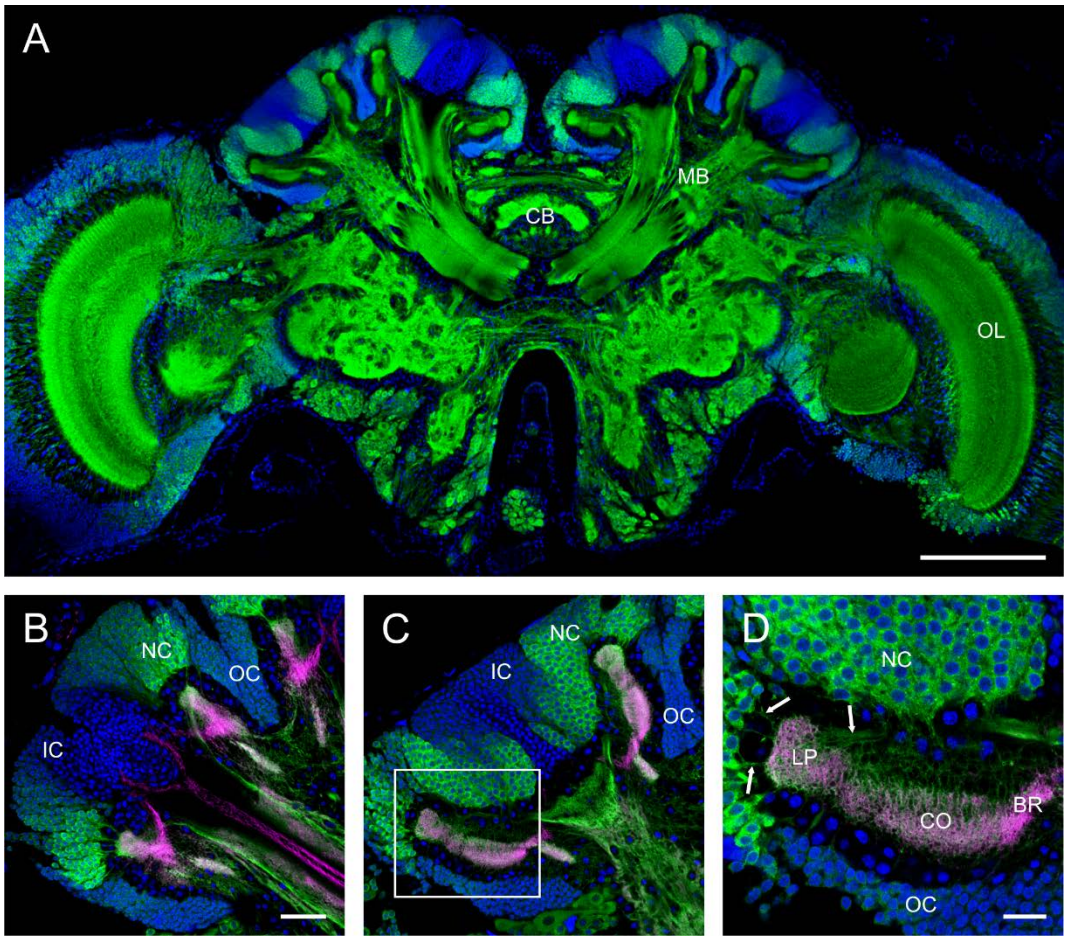


Figure 2. Distribution of pCaMKII IR in the pupal brain of *Apis mellifera*. **A:** Frontal plane overview of a pupal brain (P4) labeled with anti pCaMKII (green) and Hoechst34580 to visualize cell nuclei (blue). **B,C:** Comparison of anti-pCaMKII (green) distribution at pupal stage 3 (P3) (B) and P4 (C). Lateral calyx of the mushroom body visualized with phalloidin-labeled f-actin (magenta) and Hoechst34580 to label cell nuclei (blue). **D:** Differential anti-pCaMKII IR (green) in noncompact KCs forming the lip and collar calyx neuropil and outer compact cells. Arrows indicate anti-pCaMKII-positive dendritic branches from noncompact cells. MB, mushroom body; CB, central body; CO, collar; IC, inner compact Kenyon cells; LP, lip; NC, noncompact Kenyon cells; OC, outer compact Kenyon cells; OL, optical lobe; Scale bars = 300 µm in A; 50 µm in B,C; 20 µm in D

In both P3 and P4 pCaMKII IR was present throughout the brain at high concentrations (Fig. 2). Among KCs pCaMKII was restricted to the noncompact cells (nomenclature of KC subpopulations according to Farris and Sinakevitch, 2003), while the first born outer compact cells and the latest born inner compact cells almost totally lacked staining (Fig. 2B,C). Between P3 (Fig. 2B) and late P4 (Fig. 2C) the progress in differentiation of the MB calyx neuropil was clearly visible using f-actin-phalloidin labeling (Groh and Rössler, 2008) and pCaMKII IR was already localized in the calyx neuropil. At P4 the inner compact cell dendrites start to shape the basal ring subregion (Farris et al., 1999; Strausfeld, 2002), which is hardly identifiable in Figure 2D, and pCaMKII IR was more or less absent in this region. This indicates that the inner compact cells, which are still in the process of being born at P4, lack expression of CaMKII from the beginning at these developmental stages. High magnifications of f-actin-phalloidin labeling (magenta) in Figure 2D identified few circular postsynaptic profiles of MG, which, at this stage did not necessarily colocalize with pCaMKII (green).

In the adult brain pCaMKII IR was detectable at low levels throughout the entire brain (Fig. 3A), but staining in the MBs was always strongest and showed a characteristic differential labeling pattern (Fig. 3A,B). Slight differences in staining intensities between the medial and lateral MBs seen at this level of magnification were due to the penetration depth of the antibody and unevenness in the section (see Materials and Methods). Compared to the MBs, the optic lobes (Fig. 3A), the antennal lobes, and central complex (Fig. 3A,B,D) showed very weak IR. Similar to our immunoblotting experiments, preabsorption of pCaMKII antibody with phosphorylated blocking peptide almost completely quenched pCaMKII IR in the entire brain, whereas IR remained unaffected using preabsorption with unphosphorylated blocking peptide (data not shown).

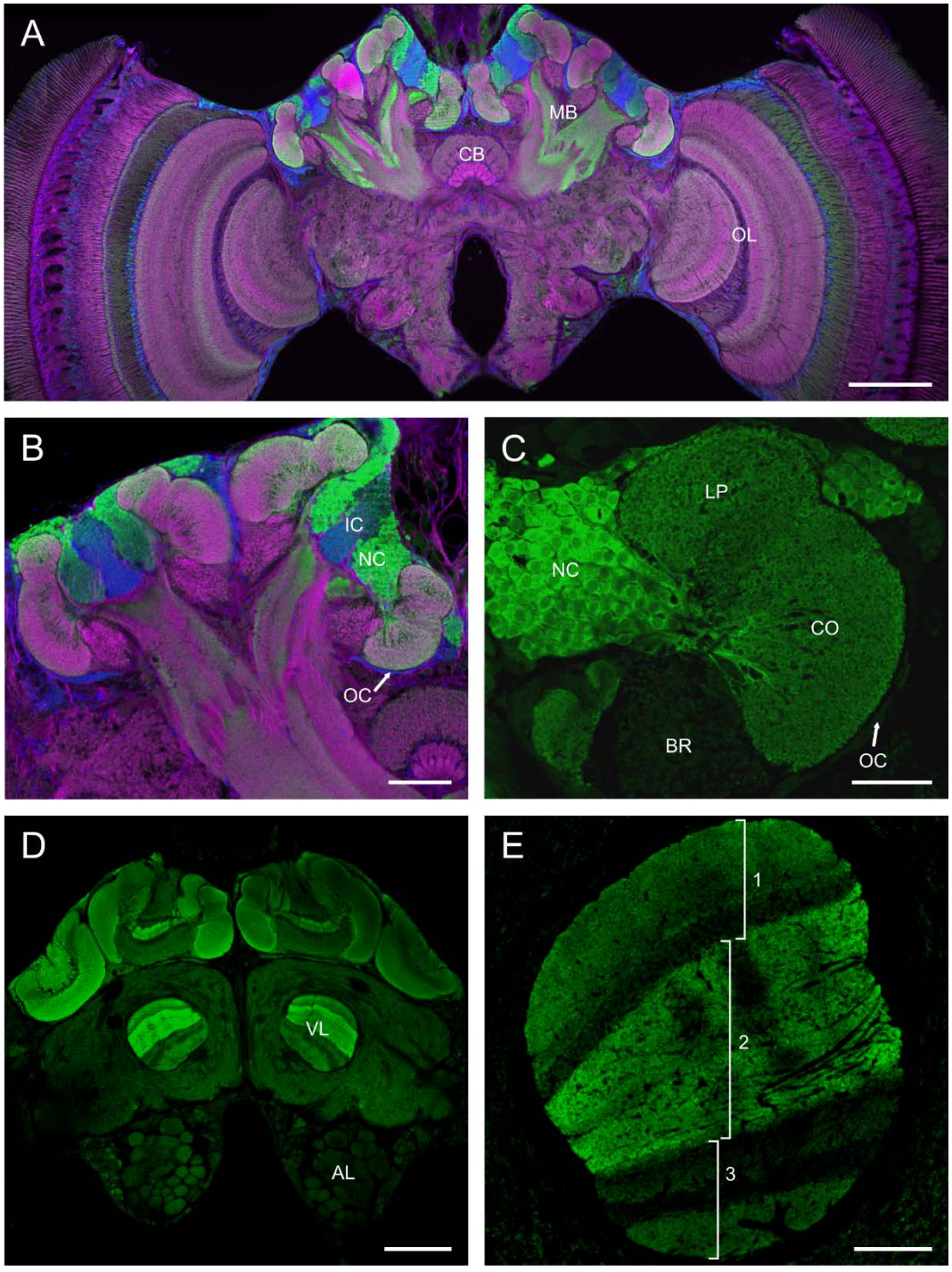


Figure 3. Distribution of pCaMKII IR in the adult brain of *Apis mellifera*. **A:** Frontal plane overview of an adult honeybee brain labeled with anti-pCaMKII (green), phalloidin-labeled f-actin (magenta), and Hoechst34580 to label cell nuclei (blue). The mushroom bodies (MB), central body (CB), and optic lobes (OL) are indicated. **B:** Overview of the MB triple-labeled with anti-pCaMKII in green, phalloidin-labeled f-actin in magenta, and Hoechst34580 in blue. **C:** MB calyx subcompartments and surrounding Kenyon cell somata labeled with anti-pCaMKII in green. **D:** Upper frontal plane overview anti-pCaMKII IR in the vertical lobes (VL) and in the antennal lobe (AL). **E:** Magnification of a VL with layers highlighted by anti-pCaMKII IR in green. The three broad subdivisions represent Kenyon cell axonal projections from the basal ring (1), lip and collar region (2) of the MB calyx, and from the outer compact Kenyon cells forming the γ lobe (3). BR, basal ring; CB, central body; CO, collar; IC, inner compact Kenyon cells; LP, lip; NC, noncompact Kenyon cells; OC, outer compact Kenyon cells; OL, optic lobe; PED, peduncle; VL, vertical lobe. Scale bars = 300 µm in A; 100 µm in B; 40 µm in C,E; 200 µm in D.

High magnification of pCaMKII IR in the MBs revealed that labeling in the basal ring (a region receiving input from both visual and olfactory projection neurons) was almost absent compared to the rest of the MB calyx neuropil (lip and collar) (Fig. 3C). Correspondingly, labeling of the inner compact cells of KC somata (KCs associated with the basal-ring neuropil; Strausfeld, 2002) was very weak compared to the noncompact cells. Similarly, IR in the outer compact layer of KCs was much weaker compared to the noncompact cells (Fig. 3B,C). However, pCaMKII IR in the noncompact cells was always brightest and showed an irregular pattern of cells, with lower IR among others with high levels of IR. In Figure 3B triple labeling of the MBs shows the distribution of KC somata (cell nuclei in blue), pCaMKII IR (green) together with f-actin-phalloidin labeling (magenta). The differential staining pattern in the calyx neuropil corresponds to the characteristic pattern of layers of KC axons in the vertical lobes (Fig. 3D). Only the regions corresponding to the collar and lip subregions of the MB calyx (according to Strausfeld, 2002) showed strong fluorescence (Fig. 3E). The other regions of the vertical lobe were only weakly labeled.

Distribution of pCaMKII within microglomeruli of the mushroom body calyx

We performed double and triple labelings to localize pCaMKII IR within individual MG, distinct synaptic complexes in the MB calyx neuropil (Groh et al., 2004) (Fig. 4). Triple labeling with anti pCaMKII, f-actin-phalloidin, and cell nuclei staining clearly revealed an overlap of pCaMKII IR with f-actin-phalloidin staining, indicating that pCaMKII is localized in dendritic spines of KCs (Fig. 4A–E) (Frambach et al., 2004). MG in the basal ring more or less completely lacked pCaMKII IR. In preparations double-labeled with anti-pCaMKII and anti-synapsin I (Fig. 4F–J) the overlap between pCaMKII and synapsin I was only marginal, giving further support to the exclusive presence of pCaMKII in postsynaptic compartments of MG and colocalization with f-actin (Fig. 4J,4G–I).

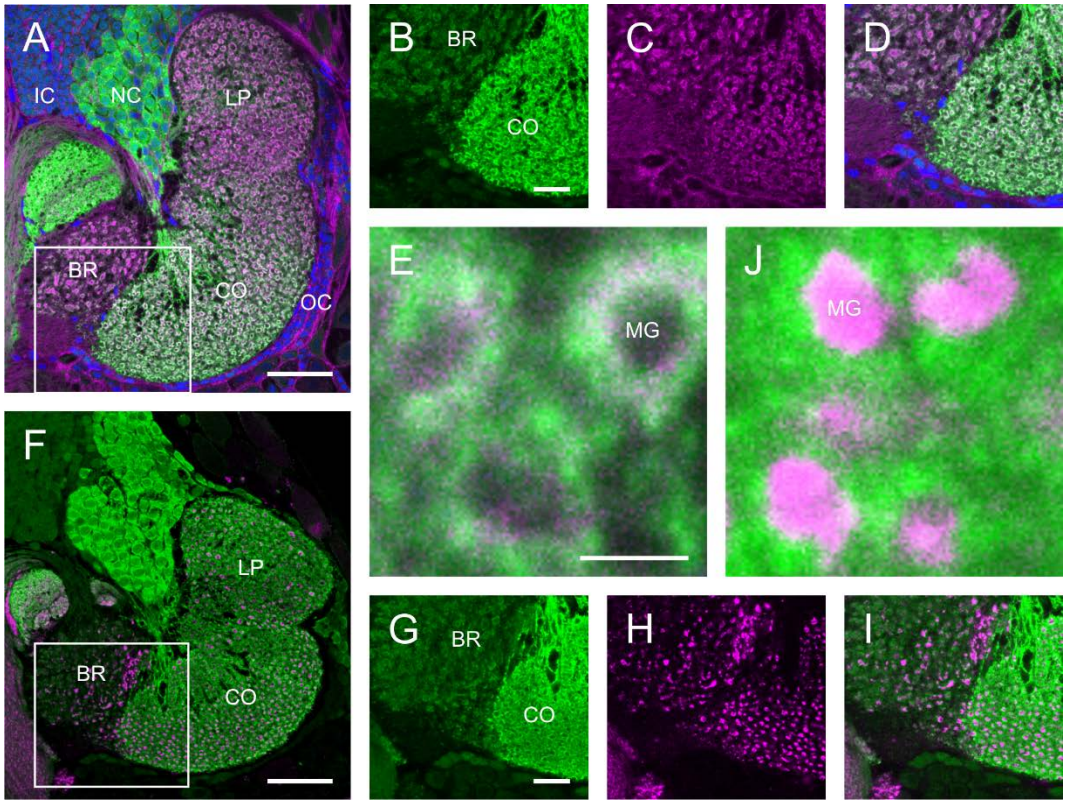


Figure 4. Localization of pCaMKII IR in MB calyx microglomeruli. **A–E:** Localization of pCaMKII IR within the MB calyx visualized by triple labeling with pCaMKII IR (green), postsynaptic phalloidin-labeled f-actin (magenta), and Hoechst34580 to highlight cell somata (blue). **F–J:** Localization of pCaMKII IR within the MB calyx shown by double labeling with pCaMKII IR (green) and presynaptic synapsin IR (magenta). A,F: Overview of the calices with rectangles indicating the position of higher-magnification images shown in B–D, or G–I, respectively. pCaMKII IR (green) is shown in B,G; phalloidin-labeled f-actin localization (magenta) in C; synapsin IR (magenta) in H; and the merged images of B,C in D, and G,H, in I, respectively. E,J: Microglomeruli magnified from the collar of D,I, respectively. BR, basal ring; CO, collar; IC, inner compact Kenyon cells; LP, lip; MG, microglomerulus; NC, noncompact Kenyon cells; OC, outer compact Kenyon cells. Scale bars = 40 µm in A,F; 15 µm in B–D,G–I; 2 µm in E,J.

Distribution of pCaMKII at different stages during adult maturation

Honeybees undergo a profound age polyethism from young individuals working inside the colony to foragers collecting food resources outside the nest (Seeley, 1982). This behavioral transition was shown to be accompanied by a volume increase of the MB calyx, and remodeling in the synaptic architecture of the MB calyx (Krofczik et al., 2008; Muenz et al., 2008; Stieb et al., 2010). To test whether the patterns and/or intensities of pCaMKII IR are associated with adult maturation and the behavioral transition from nursing to foraging, we analyzed the distribution of pCaMKII IR in brains of 1-, 8-, and 29-day-old bees. Figure 5 shows representative examples of immunolabeled brains from age-matched cohorts. At the qualitative level, comparison of the sections did not reveal any obvious differences of

pCaMKII IR in the MB calyces (Fig. 5A–C). In all cases, labeling intensity was highest in the noncompact KCs and in the corresponding lip and collar neuropil regions. In all age groups, pCaMKII IR was absent in the basal ring neuropil, in somata of the inner compact KCs, and in the outer compact layer of KCs. Similarly, the banded pattern of pCaMKII IR in the vertical lobes did not show obvious qualitative differences between the different age groups (Fig. 5D–F), and other brain regions did not show obvious changes in pCaMKII IR between different ages either. The results indicate that pCaMKII is expressed in a similar pattern from the first day of adult life to experienced forager stages. The similarity in the differential localization of the protein during adult behavioral development indicates that CaMKII in the subpopulation of (noncompact) KCs of the MBs is active throughout the entire adult life span.

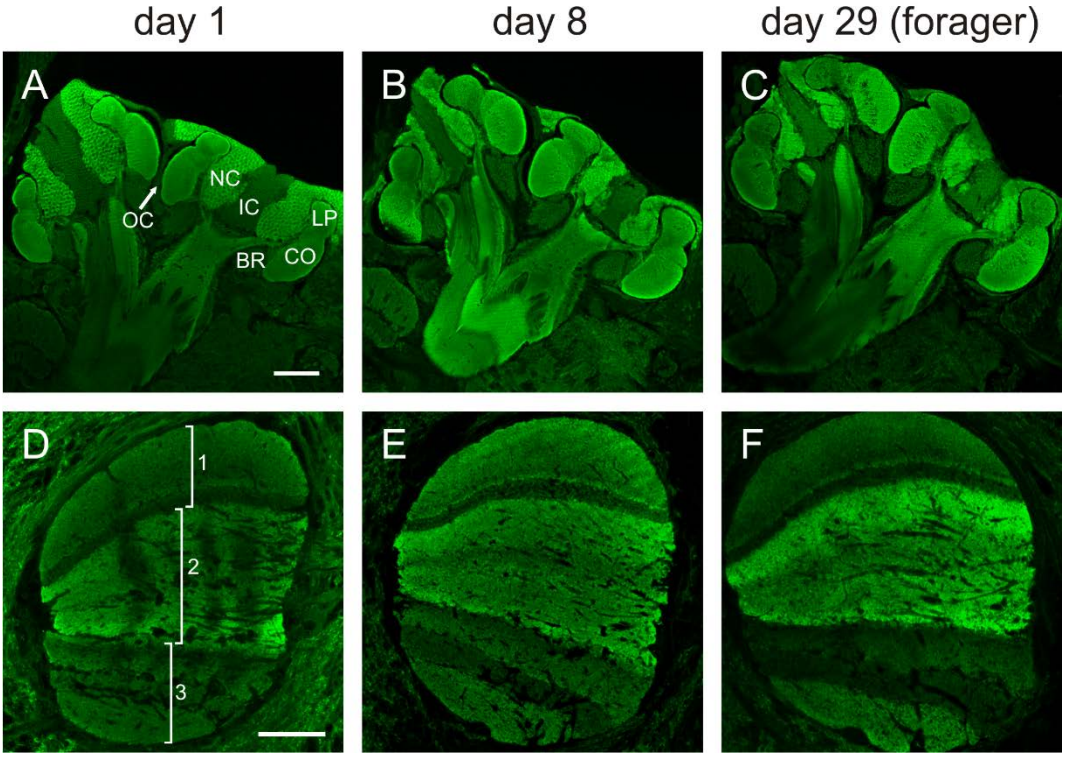


Figure 4. Comparison of pCaMKII IR during adult behavioral life-stages. **A–C:** Comparison of pCaMKII IR distribution (green) in the MB at different age stages. Indicated are calyx subcompartments (lip, collar, and basal ring) and the surrounding KC somata. **D–F:** Comparison of differential anti-pCaMKII IR distribution (green) in the vertical lobes at different age stages. The three broad subdivisions represent the Kenyon cell axonal projections from the basal ring (1), collar, and lip region (2) of the calyx and from the outer compact KCs, which form the γ lobe (3). A–F: Distribution of pCaMKII at day 1 (A,D), day 8 (B,E), and day 29 (forager) (C,F). BR, basal ring; CO, collar; IC, inner compact Kenyon cells; LP, lip; NC, noncompact Kenyon cells; OC, outer compact Kenyon cells; Scale bars = 100 µm in A–C; 40 µm in D–F.

Discussion

pCaMKII shows a differential distribution in MB, KC, and innervated synaptic neuropils in the MB calyx and lobes. Whereas detection of pCaMKII IR in the subpopulation of noncompact cells was very strong, the outer compact cells and the inner compact cells associated with the basal ring showed only low levels to absence of IR. In the noncompact cells pCaMKII IR was colocalized with f-actin-phalloidin staining in the microglomerular synapses of the calyx neuropil. Localization of CaMKII in KC dendritic spines may contribute to postsynaptic plasticity in KC dendrites of the calyx neuropil. The presence of pCaMKII IR throughout adult behavioral maturation of worker bees as revealed in age cohorts indicates that CaMKII in this subpopulation of KCs plays an important role across the entire adult life span.

Characterization of the honeybee CaMKII protein

The polyclonal antibody raised against a short amino acid sequence including phosphorylated Thr286 of CaMKIIα of human origin with 100% sequence identity in the honeybee was used in this study. The immunoblots clearly show that the antibody recognizes the phosphorylated (activated) form of CaMKII with high specificity. The antibody recognized multiple (four) bands corresponding to molecular weights from 50–65 kDa. These multiple bands are likely to represent different isoforms originating from a single alternatively spliced gene, as previously suggested for the honeybee (Altfelder et al., 1991) and other invertebrates (Cho et al., 1991; Griffith and Greenspan, 1993; Ohsako et al., 1993; Shanavas et al., 1998; Reiner et al., 1999; Griffith, 2004). Takamatsu et al. (2003) showed that in *Drosophila*, too, multiple bands of at least three CaMKII isoforms were detectable in western blot analyses (Ohsako et al., 1993). The fact that the honeybee genome contains only one copy of the CaMKII gene (Kamikouchi et al., 2000; and as also revealed by our own blast analysis), and that the antibody we used labeled multiple bands in western blot analyses in a way very similar to the pattern described in *Drosophila*, makes it very likely that the antibody detects all phosphorylated CaMKII protein isoforms. The synapsin antibody showed bands at the expected molecular weight around 74 kDa in western blot analyses, indicating that the antibody detects the similar protein as found in *Drosophila* (Klagges et al., 1996; Godenschwege et al., 2004).

General distribution of pCaMKII in the honeybee brain

The results show a wide distribution of pCaMKII in the developing honeybee brain. Investigations in M. sexta revealed an increase in postsynaptic CaMKII activation during early pupal stages (Burkert and Duch, 2006). This was shown to be correlated with dendritic filopodia collapse and rapid synaptogenesis, indicating a possible functional role of CaMKII in postembryonic development of neuronal (dendritic) circuitry. CaMKII activity in this study was shown to reflect changes in calcium influx. A study by Tamakatsu et al. (2003) showed expression of CaMKII in late-embryonic and larval stages of *Drosophila*, supporting a role of CaMKII in embryonic and postembryonic development of the neuronal circuitry in the insect nervous system. The absence of CaMKII in the inner compact KCs of the honeybee brain during pupal development indicates that these neurons may employ different molecular pathways.

Our results further show that CaMKII protein is highly concentrated and mostly restricted to the MBs in the adult honeybee brain. This observation corresponds well with the widely suggested role of the MBs in learning and memory as well as their remarkable structural plasticity (Erber et al., 1980; Heisenberg, 1998, 2003; Strausfeld et al., 1998; Menzel, 1999; 2001; Gerber et al., 2004; Davis, 2005; Menzel and Giurfa, 2006). It also brings CaMKII in a good position to potentially link changes in activity to synaptic plasticity, as shown in vertebrates (Lisman and Goldring, 1988; Lisman, 1989; Silva et al., 1992a, b; Soderling, 1993). In situ hybridizations by Kamikouchi et al. (2000) showed that expression of the CaMKII gene is mostly concentrated in MB KCs, in particular in cell bodies of the group of noncompact KCs. This is consistent with our finding that the protein is present in noncompact KC somata and in the corresponding dendritic compartments of MG within the lip and the collar subregions of the MB calyx. Interestingly, the layered axonal projections associated with the lip and the collar region in the vertical lobe (Strausfeld, 2002) also contained CaMKII. In contrast, pCaMKII IR was absent in the inner compact cells, the outer compact cells, and in the associated dendritic and axonal regions of the basal ring and MB lobes. The fact that CaMKII is enriched in one type of spiny KCs, the noncompact cells, which project to the lip and collar but not to the basal ring subregion, may indicate that only this subpopulation of KCs employs mechanisms of dendritic plasticity mediated by this protein.

The differential pattern of CaMKII IR in the MB lobes may indicate that the protein is also located in presynaptic terminals of this subpopulation of KCs (Figs. 3, 5). One interesting fact is that even staining within pCaMKII-positive layers showed differences in intensity. This may suggest an additional role of CaMKII in the plasticity of presynaptic terminals in this subpopulation of KCs and suggests a differential activation pattern within subpopulations of noncompact KCs. Furthermore, according to Strausfeld (2002) the lightly labeled layer in the lower part of the gamma lobe may be associated with basal ring KCs. If labeling in this layer were due to axonal staining it may indicate that the inner compact KCs associated with the basal ring (and/or a subpopulation of basal-ring-associated clawed KCs) may express CaMKII in axonal terminals only, but not in dendritic compartments in the MB calyx (Fig. 4). However, the extremely high packing density and the small size of synapses in the MB lobes did not allow resolution of pre- and postsynaptic compartments, as is possible in the relatively large MG of the MB calyx. Therefore, we cannot exclude that CaMKII IR in the MB lobes may well be localized in postsynaptic neurons. These intriguing results require further detailed investigations using immunoelectron microscopy.

In contrast to the rather distinct distribution of CaMKII in the MBs of the adult honeybee brain, an immunohistochemical study by Takamatsu et al. (2003) showed that CaMKII is more widely distributed in the adult *Drosophila* brain, with highest concentrations in the MB output regions, the MB lobes. In the MB lobes the differential staining patterns differ between *Apis* and *Drosophila*. This is most likely due to the representation of an additional sensory modality (visual input) in *Apis*, but also reflects pronounced differences in the neuroanatomical organization of the MB lobes between *Apis* and *Drosophila* (e.g., Strausfeld, 2002).

Cellular and subcellular localization of pCaMKII

At the subcellular level pCaMKII is concentrated in postsynaptic compartments corresponding to KC dendritic spines, in which pCaMKII and f-actin were clearly colocalized. This finding supports the potential role of CaMKII in postsynaptic (spine) plasticity (Lisman et al., 2002; Griffith et al., 2003) and possible interactions with the f-actin cytoskeleton, where it could couple functional and structural plasticity (Okamoto et al., 2009) or interfere with local translation at the level of dendritic spines (Fox, 2003). These two mechanisms have mainly been shown for vertebrates, but there are strong indications for a similar role in

invertebrates, mainly due to the high conservation of CaMKII across a wide range of taxa from human, mouse, fruit fly to the honeybee (Kamikouchi et al., 2000; Tombes et al., 2003). CaMKII may also be in a good position to mediate structural plasticity related to stable late long-term memory in MB calyx MG as shown by Hourcade et al. (2010). It may well be important to mediate changes in the MB calyx volume as most likely induced by massive dendritic outgrowth in the honeybee (Farris et al., 2001) and in the ant (Seid and Wehner, 2009; Stieb et al., 2010).

The basal ring subdivision of the MB calyx receives input from olfactory and visual projections (Ehmer and Gronenberg, 2002) and showed only very weak anti-CaMKII staining. This finding indicates that the low concentration or absence of CaMKII in inner compact KCs is independent of the input modality (olfactory or visual). Furthermore, it is known that the basal ring receives olfactory and visual input in a layered pattern from topographically and structurally divided subregions of both primary centers via different projection-neuron tracts, which send their projections each into distinct regions within the basal ring (Ehmer and Gronenberg, 2002; Kirschner et al., 2006; Galizia and Rössler, 2010). The fact that the basal ring is innervated by two modalities and shows an extremely low level of pCaMKII IR may indicate that activity mediated structural plasticity, in general, may be less expressed in this subregion of the MB calyx. This hypothesis is supported by Durst et al. (1994), showing that the basal ring of foragers only differs by 3% volume to the basal ring in age-matched nurse bees, whereas the lip and collar subregions differed by 15% and 17%, respectively. In addition to visual and olfactory input, the basal ring also receives gustatory/tactile innervation from the subesophageal-calycal tract (SCT) (Schröter and Menzel, 2003), supporting its role in multimodal processing. Another multimodal region innervated by SCT neurons is at the border between lip and collar subregion (Schröter and Menzel, 2003) which often showed slightly weaker CaMKII IR compared to the unimodal regions of the lip and the collar (Fig. 3C). A recent study by Paulk and Gronenberg (2008) showed that this region receives mixed input from both the olfactory and visual systems in the bumblebee.

In the outer compact cells (also termed “clawed KCs” by Strausfeld, 2002) pCaMKII IR was almost absent. Not much is known about the specific function of this cell cluster in comparison to others, besides its special topology and the difference in the dendritic morphology (Strausfeld, 2002; Farris et al., 2004). In contrast to all other KCs the outer compact cells send their dendrites into all subdivisions of the calyx and project their axons to

the γ lobe, which also makes them outstanding candidates for multimodal processing, besides their potential role in olfactory learning (Szyszka et al., 2008). According to Strausfeld (2002) the γ lobe itself is morphologically subdivided into three parts. It seems that, depending on the dendritic input region (basal ring, collar, or lip) of the outer compact cells, their axons form distinct layers in the reverse order of the layers formed in the vertical lobe. Interestingly, pCaMKII IR in the lowermost layer associated with the basal ring showed slightly stronger fluorescence compared to the other two layers, which is also in the reverse order compared to the axonal projections in the vertical lobe. This might as well reflect different functions of the vertical and the γ lobe.

Age-dependent patterns

The fact that the distribution of pCaMKII in brain neuropils appears to be independent of age and behavioral maturation in the honeybee suggests an essential role of this protein throughout adult life (Mayford, 2007; Abraham, 2008). The early presence of CaMKII in the MB calyx supports the recent observation that even young bees can perform olfactory classical conditioning using the proboscis extension response assay (Behrends and Scheiner, 2009). However, due to the drastic MB volume changes at the transition from nurse bees to foragers, changes in the pCaMKII concentration could also be expected at this transition (Withers et al., 1993; Durst et al., 1994; Sigg et al., 1997; Fahrbach et al., 1998; Ismail et al., 2006). On the other hand, previous studies have shown that the transition from nursing to foraging is rather flexible, as it can be induced precociously or delayed by multiple factors (Huang and Robinson, 1992, 1996; Huang et al., 1998; Pankiw et al., 1998a; Leoncini et al., 2004a). Given a potential role in structural plasticity the presence of the active protein would be required at all stages. In some preparations we saw a trend for an increase at older stages in the vertical lobe at the qualitative level (Fig. 5D–F). However, quantitative measurements of intensity ratios across individuals failed to show significant differences because of too-high variances in staining intensities. Potential differences in the amount of protein present at different stages may be determined by a quantitative comparison of the ratio between the unphosphorylated (inactive) and active form of the protein using a combination of immunocytochemistry and quantitative immunoblots. Furthermore, the high sensitivity of the antibody in immunostainings of the brain may mask slight changes in concentration of the activated state.

One important future experiment will be to systematically test whether increased levels of pCaMKII follow associative learning or sensory activation protocols. Experiments by Lent et al. (2007) in the cockroach indicate an increase of pCaMKII in the MB calyces after unilateral multimodal training of split brain insects. The differential distribution of CaMKII in different populations of KCs in the honeybee offers opportunities for manipulations to dissect their function for different learning and memory-associated tasks and potentially sensory activity and age-related alterations in the neuronal circuitry.

Acknowledgements

We thank Dorothea Eisenhardt, Manfred Schartl, and Johannes Haydn for help and technical support with immunoblotting experiments, Christina Scholl and Saskia Eck for assistance with control experiments, Erich Buchner for the kind gift of anti-synapsin antibodies, and Dirk Ahrens for expertise and support in beekeeping.

Grant sponsor: HFSP; Grant numbers: RGP42/2007, SFB 554/A8, SPP 1392

Chapter III

Sensory reception of the primer pheromone ethyl oleate

Abstract

Social work force distribution in honeybee colonies critically depends on subtle adjustments of an age-related polyethism. Pheromones play a crucial role in adjusting physiological and behavioral maturation of nurse bees to foragers. In addition to primer effects of brood pheromone and queen mandibular pheromone - both were shown to influence onset of foraging - direct worker-worker interactions influence adult behavioral maturation. These interactions were narrowed down to the primer pheromone ethyl oleate, which is present at high concentrations in foragers, almost absent in young bees and was shown to delay the onset of foraging. Based on chemical analyses, physiological recordings from the antenna (electroantennograms) and the antennal lobe (calcium imaging), and behavioral assays (associative conditioning of the proboscis extension response), we present evidence that ethyl oleate is most abundant on the cuticle, received by olfactory receptors on the antenna, processed in glomeruli of the antennal lobe, and learned in olfactory centers of the brain. The results are highly suggestive that the primer pheromone ethyl oleate is transmitted and perceived between individuals via olfaction at close range.

With kind permission from Springer Science+Business Media:

Muenz TS, Maisonnasse A, Le Conte Y, Plettner E, Rössler W. 2012. Sensory reception of the primer pheromone ethyl oleate. Naturwissenschaften 99:421-425

The original article is available at:

http://link.springer.com/article/10.1007/s00114-012-0909-1

Introduction

Division of labor is a most astonishing characteristic of insect societies. In honeybee colonies, worker bees exhibit a pronounced age-dependent polyethism. Young individuals take over in-hive duties, whereas bees around the third week of adult life start to forage for nectar, pollen, and water (Lindauer, 1952). Timing of this behavioral transition is crucial for colonies to respond to changing environmental conditions. Adult behavioral maturation goes along with physiological changes and is associated with an increased juvenile hormone (JH) titer (e.g., Robinson, 1992).

How do individual bees acquire information about the status of the colony or "know when to become a forager"? Two multicomponent primer pheromones—the brood pheromone (BP) and the queen mandibular pheromone (QMP)—influence the colony at various behavioral and physiological levels (Le Conte and Hefetz, 2008) and are likely candidates to maintain an appropriate equilibrium between nurse bees and foragers. As this distribution is essential to maintaining appropriate brood development, it is not surprising that BP not only influences the number of pollen collecting foragers (Pankiw et al., 1998b) but also alters the onset of foraging (Le Conte et al., 2001). QMP was shown to induce both primer and releaser effects on worker bees and to affect onset of foraging by altering JH levels (Pankiw et al., 1998a). In addition to these two well-studied primer pheromones, direct worker–worker interactions were suggested to influence nurse–forager transition. Rösch (1930) used an elegant approach to deprive honeybee colonies of all foragers, which induced precocious onset of foraging in young nurse bees. Rösch's experiment was the first indication for a potential forager signal. This was further substantiated by blocking physical contact between young bees and foragers, which revealed inhibitory effects of foraging bees on behavioral maturation of young bees (Huang et al., 1998). Later, cuticular extracts of foraging bees were shown to delay the onset of foraging and to alter the sucrose responsiveness of individual bees (Pankiw, 2004). Finally, Leoncini et al. (2004a) showed that ethyl oleate (EO) is abundant in the honey crop of only foraging bees. In experiments controlling for brood and QMP, they were able to show that EO inhibits the onset of foraging. This led to the identification of EO as a primer pheromone transmitted by foragers. Interestingly, EO was also found as a component of the pheromone blend of queens and larvae and, therefore, classified as a colony pheromone (Keeling and Slessor, 2005; Slessor et al., 2005).

The mode of transmission and reception of EO in honeybee colonies, however, is still not well understood. As physical contact between workers is necessary to influence the onset of foraging (Huang et al., 1998) and EO was found in the honey crop, trophallaxis was suggested as the major mode of transmission (Leoncini et al., 2004a). In the present study, we provide evidence that EO is most likely transmitted as a low-volatility signal at close range or during contact and perceived via the olfactory system.

Material and methods

The following are general procedures of all methods used. Further technical details are provided in the supplementary material.

EO quantification

To evaluate sources for transmission, EO was quantified in different body regions (head, thorax, abdomen, cuticle, honey crop content, honey crop envelope, and regurgitate) of foraging bees collected at the hive entrance. Samples were prepared in hexane with two internal standard solutions, fractioned to separate ester fraction, and injected into a gas chromatograph with a flame ionization detector and capillary column. EO identification and quantification was based on retention times of synthetic EO (Sigma-Aldrich, France) and comparison of internal standard area to the compound area. EO confirmation was done by mass spectrometry. EO amounts were analyzed with a Kruskal–Wallis test ($\alpha < 0.05$) followed by Mann–Whitney U tests ($\alpha < 0.05$).

Electroantennography

Electroantennography recordings were used as an indicator for EO reception by antennal olfactory receptor neurons. Isolated flagella from individual bees were placed on differential electrodes connected to a Syntech-based (Hilversum, the Netherlands) setup. Stimuli (diluted EO or dist. hexane) were presented in a pulse compensation setup directing a continuous humidified air flow to the antennae. To simulate temperature conditions inside the hive and increase EO volatility, the airstream was heated to ~36 °C. For recording and analyses, Autospike 1.0 and a customized Labview 6.1-based program (C. Kleineidam) were

used. Data were tested for normal distribution with Kolmogorov–Smirnov test ($\alpha < 0.05$) followed by paired T test ($\alpha < 0.05$).

Appetitive conditioning

To investigate whether EO is perceived and transmitted to higher brain centers, we used a well-established associative conditioning paradigm, the proboscis extension response (PER) (Bitterman et al., 1983) with EO as the conditioned stimulus and a sucrose reward as unconditioned stimulus. Stimuli (diluted EO or dist. hexane) were presented as air puffs. PER response ratios at the fifth training trial were analyzed using Fishers exact test ($\alpha < 0.05$).

Calcium imaging

A pilot calcium imaging experiment was performed to probe whether EO is processed in the antennal lobe (AL). To monitor output activity of AL glomeruli, projection neurons were retrogradely filled with a calcium indicator. For stimulation, a heated Teflon dummy was loaded with diluted EO, dist. hexane or left blank. The dummy (Brandstaetter et al., 2011) was moved toward the bee's antennae without physical contact to mimic a nearby EO emitting bee.

Results

EO localization

GC/MS analyses revealed a significant difference in the distribution of the absolute EO amount in three body sections of foragers (Table 1) ($n = 30$, $H = 16.41$, $df = 2$, $P < 0.001$). Considering the total amount per section, EO was predominantly present in the abdomen compared to the head ($Z = -0.302$, $P < 0.01$) and the thorax ($Z = -3{,}780$, $P < 0.001$). The EO amount in the head was significantly higher than in the thorax ($Z = -1.965$, $P < 0.05$). About 26 % of the total EO amount was found on the cuticle, while no trace of EO was found in the nectar inside the honey crop and in the regurgitated nectar. EO was only found in the honey crop's envelope representing 12 % of the total amount of EO in a forager.

Table 1. EO localization

Body part	Mean amount of EO (ng ± SE)	EO per forager (%)
Head	19.75±3.79	22.60
Thorax	7.52±2.62	8.60
Abdomen	58.33±4.91	66.74
Honey crop envelope	8.89±4.03	12.20
Honey crop content	0.00	0.00
Regurgitated nectar	0.00	0.00
Cuticle	16.75±3.74	25.77

GC/MS analysis of head, thorax, and abdomen shows significant differences in ethyl oleate distribution with the highest quantities in the abdomen. No trace of ethyl oleate was found in the nectar (inside the honey crop or regurgitated), but high concentrations were located in the honey crop envelope. About one quarter of the total ethyl oleate amount was found on the cuticle

EO reception via the olfactory pathway

Stimulation with EO diluted in dist. hexane evoked significantly ($n = 37$, $P < 0.001$) stronger responses than stimulation with solvent only (Fig. 1A, B). To probe whether EO is processed in the AL, we performed calcium imaging measurements and close range stimulation with an EO loaded Teflon dummy. These first recordings revealed calcium activation in projection neurons in the upper AL (most likely in T1 associated glomeruli) (Fig. 1C, D). No calcium activation was detectable in response to solvent and the unloaded heated dummy.

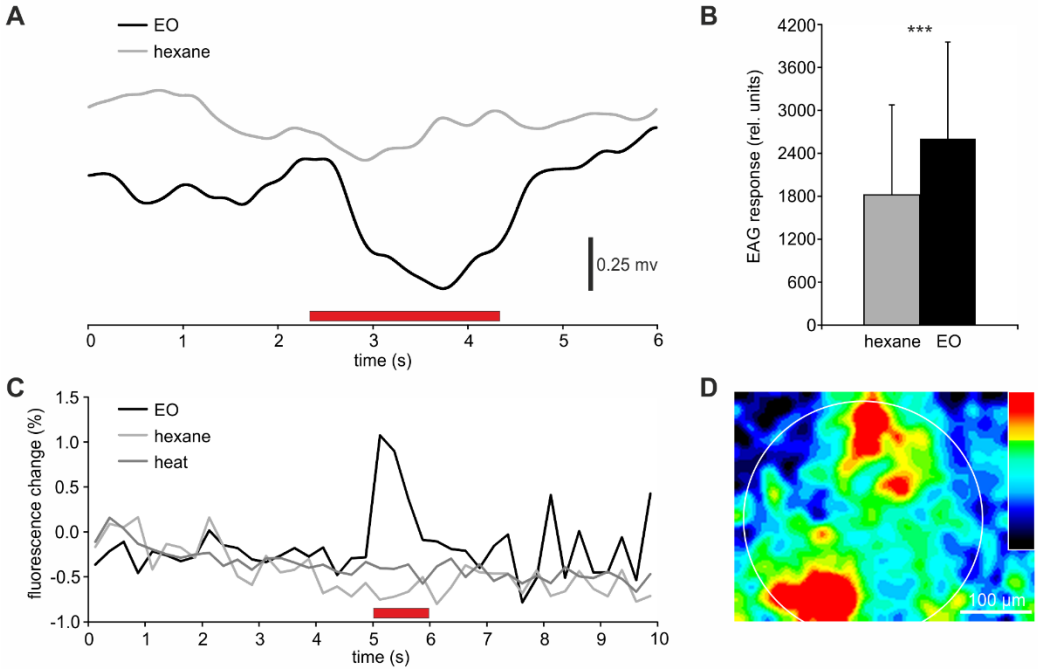


Figure 1. Physiological recordings show that ethyl oleate is received and processed via the olfactory system. **A,B** Typical electroantennography recordings show clear responses to stimulation with ethyl oleate (red bar) in contrast to solvent only. **C** Mean neuronal activity measured in the antennal lobe shows a strong response to ethyl oleate loaded dummy stimulation (red bar). No activity changes were recorded during control stimulation. **D** Activity pattern in the antennal lobe (white circle) during ethyl oleate stimulation reveal activity spots (red) associated with glomeruli in the upper antennal lobe. EO ethyl oleate, EAG electroantennography; heat scale ($\Delta F_{340}/F_{380}$) ranges from −0.7 to 1 %

Olfactory perception and learnability of EO

Using EO as the conditioned stimulus during an associative learning paradigm revealed a shallow learning curve (Fig. 2) for training with EO compared to non-pheromonal odors (e.g., Bitterman et al., 1983). However, in comparison to the control stimulus (dist. hexane only, n = 90), bees were clearly able to learn to associate a dilution of EO in dist. hexane (n = 128) with the sucrose reward ($P < 0.001$).

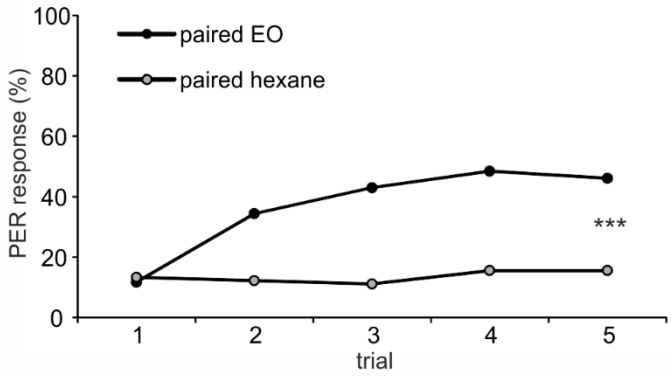


Figure 1. Olfactory conditioning with ethyl oleate. In a five-trial training paradigm, bees were significantly able learn to associate ethyl oleate with a sucrose reward in contrast to solvent only

Discussion

The primer pheromone EO is an important social regulator produced by foraging bees that delays behavioral maturation of nurse bees (Leoncini et al., 2004a). In contrast to earlier assumptions, our results show that EO is not present in regurgitated nectar of foragers but instead is highly abundant on the cuticle, which makes it an easily accessible olfactory signal for close range transmission within the hive. The present physiological and behavioral measurements demonstrate that EO is received, perceived, and learned as a close range olfactory stimulus.

Adaptive adjustments in division of labor depend on the ability of worker bees to respond to the status of the colony. It seems likely that the necessary cues are perceived during patrolling within the hive (Lindauer, 1952). Earlier experiments suggested that physical contacts like licking, trophallaxis (Free, 1965), or antennation mediate potential signals (e.g., Huang et al., 1998). The association of EO with the honey crop of foraging bees (Leoncini et al., 2004a) led to the hypothesis that EO may be one signal transmitted by trophallaxis. Our present results show that neither nectar inside the honey crop nor regurgitate contains EO. Instead EO is present only in the honey crop envelope. The cuticle contains about a quarter of the total EO amount in foragers, representing the main EO source for interindividual transmission and indicating a prominent role of olfaction in the reception of EO, in contrast to gustation or ingestion as suggested earlier (Leoncini et al., 2004a). Our physiological measurements and PER conditioning show that EO is received and perceived via the olfactory pathway. Also, EO biosynthesized in the gut of foragers accumulates on the cuticle, from where it evaporates (Castillo et al., 2012a). These observations further support the idea of EO acting as a short range/contact pheromone via the olfactory pathway. Its low volatility explains why a double-screen wire mesh usually prevents its action (e.g., Huang et al., 1998). The fact that EO acts via olfaction, however, does not exclude that it may additionally act via gustation, but its absence in regurgitate excludes trophallaxis as the mode of transmission.

How is the primer effect of EO expressed? For the primer pheromone QMP, it was shown that one component, homovanillyl alcohol (HVA), shares structural similarity with dopamine. Experiments led to the assumption that HVA may directly interact with brain dopamine receptors which in turn influences the behavior of young worker (Vergoz et al., 2007; Beggs et al., 2007; Beggs and Mercer, 2009). However, it is still unclear how HVA is transferred into

the brain. Whether EO acts solely via the olfactory pathway to alter adult maturation or whether alternative routes like effects on biogenic amines or juvenile hormone levels (Wagener-Hulme et al., 1999; Huang and Robinson, 1992) are involved needs to be shown. It is also possible that EO reduces basal levels of behavioral activity resulting in a delay of first orientation flights that are needed to promote neuronal and behavioral maturation.

Acknowledgments

We appreciate the help of Jan Kropf with electroantennography recordings, Dominique Schmitt with olfactory conditioning, Andreas Brandstaetter and Christoph Kleineidam for technical expertise with the dummy stimulation and lab view programs, Dominique Beslay for chemical analyses, and Dirk Ahrens for beekeeping support. Funding was provided by Human Frontier Science Program (RGP0042/2007)

Online Supplement

Detailed Material and Methods

EO Quantification

To evaluate sources for transmission, EO was quantified in different body regions (head, thorax, abdomen, cuticle, honey crop content, honey crop envelope and regurgitated nectar) of foraging bees collected at the hive entrance of a typical field colony (headed by a naturally mated queen) and immediately frozen at -20°C.

To gather 10 samples of each honeybee body section (5 body sections per sample), the heads, thoraxes and abdomens of 50 foragers were dissected. For honey crop content and honey crop envelope analyses, the abdomens of 6 samples (5 foragers per sample) were dissected by making an incision to remove the crop. The honey crop content was evacuated using a syringe and separated from the honey crop envelope. Six samples (5 foragers per sample) of nectar regurgitation were obtained with a microcapillary while applying pressure to the abdomen with forceps. Ten samples (5 foragers per sample) of cuticular extracts were

prepared by solvent rinsing. As a control, 10 samples (5 foragers per sample) were analysed for the total EO amount.

Samples were prepared in 1.9 mL of iso-hexane (2-methylpentane) with 100 µL of two internal standard solutions at 10 ng/µL (arachidic acid methyl ester and methyl heptadecanoate, Sigma-Aldrich, France) by either crushing with a glass rod for 2 min at 0°C and centrifuging for 20 min at 4°C (2,500 g) or by rinsing bees for 1 min at 0°C for EO cuticular extracts analysis.

The supernatant was collected and applied to a silica column (Pasteur pipette filled with 0.71 g silica gel 60, 40–63 µm particle size, and a 230–400 µm mesh at the bottom). The first fraction was eluted in 3 mL of a solvent (98.5% iso-hexane, 1.5% diethyl ether), the second fraction containing EO was eluted in 3 mL of a second solvent (94% iso-hexane, 6% diethyl ether). 1 mL of this fraction was concentrated to 10 µL under a nitrogen stream, and 1 µL was injected (in split mode) into a gas chromatograph (2014, Shimadzu, Japan) with a flame ionization detector and capillary column Omegawax 100 (10 m x 0.10 mm, 0.10 µm film thickness). Hydrogen was used as carrier gas (column flow 0.52 mL/min). Oven temperature was set 90°C for 1 min, raised to 195°C at 40°C/min, stabilized for 3 min, then augmented to 210°C at 1°C/min, stabilized again for 2 min, then increased to 270°C at 40°C/min and held at 270°C for 3 min. Identification and quantification of EO was based on retention times of synthetic EO (Sigma-Aldrich, France) and comparison of internal standard area to the compound area using the gas chromatography solution program (Shimadzu, Japan). EO confirmation was done by mass spectrometry (CP2010, Shimadzu, Japan) operated in the electron impact mode (70 eV) with continuous scans (every 0.2 s) from a mass to charge ratio (m/z) of 70 to 400 amu.

EO amounts were analyzed with a Kruskal-Wallis test ($\alpha < 0.05$) followed by Mann–Whitney U tests ($\alpha < 0.05$).

Electroantennography (EAG)

EAG recordings were used as an indicator for EO reception by antennal olfactory receptor neurons. Isolated flagella from individual bees (n = 37) were placed in electrode gel (Spectra 360, Parker, NJ) on differential electrodes. The recording electrodes (Syntech, Hilversum, The Neverlands) were connected to a preamplifier (Universal AC/DC probe 10, Syntech, Hilversum, The Neverlands) and data acquisition interface (IDAC-USB, Syntech, Hilversum,

The Neverlands). Stimuli were presented in a pulse compensation setup directing a continuous humidified air flow (0.5 L/min; Thomafluid KDM 65, Reichelt, Heidelberg, Germany) to the antennae. Airflow was directed through an empty or filter paper (5 mm x 40 mm) containing 1 mL syringe either loaded with 10 µl distilled hexane or diluted EO in dist. hexane (10^{-3}). To simulate temperature conditions in the hive and increase the volatility of EO the airstream was heated to ~36°C upon leaving the syringe (custom built heat exchanger connected to a water bath; Thermostat MGW, Lauda). For recording Autospike 1.0 (Syntech Hilversum, The Netherlands) was used. The integral of responses was quantified from the time of stimulus onset to stimulus offset using a customized Labview 6.1 based program (C. Kleineidam). Data were tested for normal distribution with Kolmogorov-Smirnov test ($\alpha < 0.05$) followed by paired T-Test ($\alpha < 0.05$).

Appetitive conditioning

To investigate whether EO is perceived and transmitted to higher brain centers, we used a well-established associative conditioning paradigm, the proboscis extension response [PER] (Bitterman et al., 1983) with EO as conditioned stimulus (CS) and sucrose reward as unconditioned stimulus (US). Honeybees were collected at the hive entrance, cooled on ice and harnessed in plastic tubes allowing free movements of the proboscis and antennae. In the late afternoon, before being placed in a dark incubator (30°C, ~75% rel. humidity), bees were fed ad libitum with 50% water/sucrose solution. The next morning each bee received 5 training trials (10 min intervals). Each trial consisted of 4 s CS and 4 s US (50% water/sucrose) presentations with 2 s overlap (2 s inter-stimulus interval). Stimuli we presented like in EAG recordings as heated air puffs of either diluted EO in dist. hexane (10^{-3}) (n = 128) or dist. hexane (n = 90). Data was analyzed using Fishers Exact test ($\alpha < 0.05$) for the PER response ratios at the fifth training trial.

Calcium imaging

We performed a pilot calcium imaging experiment to probe whether EO is processed in the antennal lobe (AL). To monitor output activity of AL glomeruli, projection neurons were retrogradly filled with Fura-2-dextrane. A heated teflon dummy (40°C) was loaded with 20 µL of EO diluted in dist. hexane (10^{-3}), pure dist. hexane or empty. The dummy was moved towards the bee's antennae without physical contact (2-3 mm distance) to mimic a close EO

emitting bee. For further technical details on preparation, image acquisition, and stimulation see Brandstaetter et al. (2011).

General Discussion

In the present thesis, division of labor in honeybee workers was investigated with a special focus on neuronal plasticity processes in the mushroom body. This aimed at an understanding of neuronal mechanisms promoting age-related polyethism. One major objective of this study was to provide a detailed description of cellular and subcellular changes in the synaptic architecture of the mushroom body calyx to understand the basis for volume changes in correlation with behavioral maturation. This part of my thesis revealed that the mushroom body calyx expresses a continuous volume expansion including a substantial increase during the first week of adult life driven by dendritic growth in Kenyon cells and followed by pruning of microglomerular complex synapses during the transition from indoor tasks to foraging. These data provide a new framework of neuronal plasticity processes that allowed to investigate whether a primer pheromone, ethyl oleate, that was shown influence behavioral maturation, also affects structural neuronal plasticity processes in the mushroom body calyx. Manipulations of ethyl oleate concentrations in artificial triple cohort colonies did not reveal a direct connection between the primer pheromone and neuronal plasticity. However, the results on synaptic maturation clearly confirmed the general data on natural maturation processes in the mushroom body calyx.

As changes in the dendritic network of the mushroom body intrinsic neurons, the Kenyon cells, were identified as the main contributor for volume expansion and driver of neuronal plasticity in the mushroom body calyx the question arose which molecular pathways could be involved. For this the protein calcium/calmodulin-dependent kinase II (CaMKII) was analyzed in the mushroom bodies. The results show a distinct localization of CaMKII restricted to the noncompact Kenyon cells. As CaMKII is known for its role in mediating learning and memory processes, for example long term potentiation, and has the potential to directly interact with the postsynpatic cytoskeleton, this protein is a likely candidate to mediate structural plasticity in at least two subregions, the lip and the collar, of the mushroom body calyx. The absence of this protein in the basal ring indicates that other plasticity mechanisms are likely to be at work in this subregion.

The primer pheromone ethyl oleate was shown to be a factor regulating behavioral maturation. As high concentrations are only found on foragers, it is thought to serve as an

indicator for the colony work force distribution. This study revealed that ethyl oleate is not located inside the honey crop, but only in the honey crop tissue (envelop) from where it is likely to be transported to the cuticle. Together with neurophysiological measurements at different levels along the olfactory pathway as well as olfactory conditioning clearly showed that ethyl oleate can be received, perceived and learned as a short range olfactory stimulus.

Neuronal plasticity in the context of division of labor under natural conditions

Division of labor among worker honeybees in form of an age-related polyethism is based on individual behavioral maturation, which in turn clearly relies on the adjustment of physiological and anatomical prerequisites that workers need to fulfill their respective tasks. But not only glandular tissue, for example wax glands, develop in correlation to the corresponding task (for review see Winston, 1987), also sensory systems as well as higher-integration centers in the brain need to adapt for new sensory stimuli or behavioral patterns. The mushroom bodies as multimodal integration and learning and memory centers represent an ideal substrate to analyze neuronal plasticity in correlation to behavioral maturation. In line with earlier studies on the honeybee (Withers et al., 1993, 1995; Durst et al., 1994; Farris et al., 2001; Groh et al., 2012), but also on other hymenopteran insect species like the carpenter ant *Camponotus floridanus* (Gronenberg et al., 1996), the desert ant *Cataglyphis bicolour* (Kühn-Bühlmann and Wehner, 2006), and the paper wasp *Polybia aequatorialis* (O'Donnell et al., 2004) my thesis revealed a volume increase of all mushroom body calyx subregions during adult maturation.

The novel and important aspect of my thesis is, that volume measurements were combined with high resolution analyses on cellular and subcellular processes underlying volume plasticity. This allows, for the first time, to disentangle volume dependent and volume-indepenedent cellular processes during adulkt maturation. During the substantial volume increase in the first week of adult life, the total numbers of synaptic complexes (microglomeruli) within the lip and collar subregion of the calyx remain constant. Immunolabeling of the Kenyon cell dendritic network was able to assign the calyx volume increase to a massive outgrowth of the Kenyon cell dendrites. This was already proposed in a random labeling Golgi study by Farris et al. (2001) showing increasing branching of Kenyon cell main dendritic arborizations during the transition from nursing to foraging. Previous studies suggested that the initial volume increase is independent of social interactions or

sensory stimuli (Withers et al., 1995; Fahrbach et al., 1998) indicating an internal program as its driving force. At the behavioral level, workers at this stage express a greatly reduced repertoire together with low general activity and their exclusive task of being cell cleaners, a task that is performed by older bees as well on the side (Seeley, 1982). After this initial period of maturation, during the second week of adult life, workers move over extended distances within the nest and start feeding the brood (for review see for example Winston, 1987). During this period, the likelihood of performing first short orientation or defecation flights increases (Capaldi et al. 2000). Within this period, the mushroom body calyx volume is still increasing, but, interestingly, the total number of microglomeruli in the lip and collar starts to decrease. This indicates two different plasticity mechanisms: (i) pruning of projection neuron terminals similar to observation made in *Cataglyphis* desert ants (Seid and Wehner, 2009), and (ii) continued outgrowth of Kenyon cell dendrites as the main contributor to volume increase. The general pattern of these two plasticity processes remains and is particular pronounced during the transition to foraging after the third week of adult life. Ultrastructural studies have shown that individual microglomerular complexes undergo a synaptic reorganization during the overall period which results in an increase of synaptic divergence by about 33% at the level of individual microglomeruli (Groh et al. 2012). As a net result, age-related behavioral maturation leads to an increased neuropil volume of the mushroom body calyx but a net decrease in total microglomeruli numbers in foragers when compared to indoor workers. Due to the temporal correlation of first flight activity and neuronal plasticity, sensory exposure is very likely to act as the main trigger during this phase inducing pruning of microglomeruli associated microcircuits. This is supported by artificial light exposure studies in *Cataglyphis* ants (Stieb et al., 2010; 2012), and in the honeybee (Scholl et al., 2014) showing that sensory stimulation (light) induces changes in the microglomeruli densities in the visually innervated collar.

Plasticity in the first week of adult life supports the assumption that young worker bees go through a prolonged neonatal neuronal development which is mainly characterized by outgrowth of Kenyon cell dendrites. This could be interpreted as an experience expectant (Fahrbach et al., 1998) period as it occurs before the bees are able to express their full behavioral repertoire. This is likely to go along with further competitive plastic changes in the mushroom body like the pruning of microglomeruli following changes in sensory exposure and activity.

Neuronal plasticity in the context of division of labor under manipulated conditions

Division of labor among workers is not purely age-related but in fact modulated by various external factors enabling colonies to perform a flexible shift of work forces from indoor to outdoor tasks depending on the actual needs of the colony. Especially variations in the colonies' demography have been shown to affect division of labor by delaying, accelerating or reverting individual behavioral development (for review see for example Robinson, 1992; Page et al., 2006; Johnson, 2010). For instance workers as young as 5 days have been described to get involved in foraging tasks when all foragers are removed from the colony (Rösch 1930). It has been established that the presence of foragers is a behavioral maturation influencing signal (Huang et al., 1998) that is communicated in the colony through the concentration of the primer pheromone, ethyl oleate, found in high concentrations only in foragers (Leoncini et al., 2004a; b). Ethyl oleate is biosynthesized from natural occurring ethanol-containing nectar, probably as a detoxification product, in the esophagus (Castillo et al., 2012a) and stored in the honey crop (Leoncini et al., 2004a). As only foragers get in contact with fermented nectar the concentration of ethyl oleate in the colony represents an excellent measure how many workers are involved in foraging tasks. In consequence, it has been demonstrated that artificially applied additional ethyl oleate delays the onset of foraging in workers (Leoncini et al., 2004a). In my thesis I show that ethyl oleate is not found in the content of the honey crop. Therefore, we have to reject the hypothesis that ethyl oleate is passed on via trophallaxis as suggested earlier (Leoncini et al., 2004a). Instead it was detected in the honey crop tissue (envelop) from where it is transported via an unknown pathway to the cuticle (Castillo et al., 2012a; b) and received as a short-range olfactory stimulus when evaporating from the surface. One interesting aspect emerging from this part of my thesis is that ethyl oleate can be associated with sugar reward and therefore learned and memorized. This aspect needs to be incorporated in future models of regulation in the division of labor. It is a likely assumption that neuronal plasticity, in particular changes in the synaptic architecture of the mushroom body calyx, are not exclusively age-related (see first paragraph of discussion), but can rather be modulated by factors that are known to influence behavioral maturation. I tested this hypothesis for the primer pheromone ethyl oleate. At the behavioral level, the results, however showed that the behavioral phenotype (delayed onset of foraging) was not always consistent and may be strongly influenced by other environmental variables like weather conditions. Furthermore,

the results did not show a direct correlation between the increased exposure to this pheromone and effects on neuronal plasticity. This excludes a direct effect of ethyl oleate on cellular processes underlying neuronal plasticity in the mushroom bodies. I hypothesize that the manipulation of ethyl oleate concentration in my experiments was not precise enough as it was not possible to control for the (varying) natural occurrence of ethyl oleate in foragers (Castillo et al., 2012b). Furthemore, distribution of ethyl oleate within the colony should use a more efficient mechanisms considering close range chemoreception. As a general result, however, the obtained neuronal data from this part of my thesis clearly support my observations on natural neuronal maturation processes. This is an important aspect as two different honeybee races (*Apis mellifera carnica* and *Apis mellifera ligustica*) were used in the two experimental series, which allows to conclude that the fundamental processes of neuronal maturation are very similar.

It appears plausible that particular changes in the neuronal network are necessary to allow workers to actually perform different behavioral repertoires in the context of flexible division of labor. In turn this implies that those changes can be induced by behavioral influencing factors such as pheromones. For example workers within the first week of adult life are generally assumed to be unable to express associative olfactory learning, due to their extremely low sucrose responsiveness (Pankiw and Page, 1999). However, on the other hand precocious foraging can be induced during this period (for review see for example Robinson, 1992). Associative olfactory learning seems to a prerequisite to accomplish foraging tasks. It is therefore not surprising that sucrose responsiveness alters with increasing age, being low in (nectar) foragers, and shows a correlation to juvenile hormone (Pankiw and Page, 2003). Exposure to brood and queen mandibular pheromone as known factors to influence behavioral maturation (ethyl oleate was not tested in this regard), should therefore also modify sucrose responsiveness, which they actually do (Pankiw and Page, 2003). Changes in the synaptic architecture of the mushroom body calyx, which is strongly involved in learning and memory tasks, could be the last step in the chain of events that allow for flexible division of labor but still needs to be proven.

Mechanisms underlying structural neuronal plasticity in the mushroom bodies

As demonstrated in this thesis, structural neuronal plasticity in the mushroom body calyx is an important mechanism occurring during adult maturation, but also during associative

learning and long-term memory formation (Hourcade et al., 2010). Even though many factors are known to influence behavioral maturation and have in turn been suggested to correlate to structural changes in the mushroom body, the actual molecular mechanisms that finally induce plastic changes on a cellular and subcellular level are only poorly understood. In this thesis I therefore investigated the protein calcium/calmodulin-dependent kinase II (CaMKII) which is well known for its role in long term potentiation (e.g. recruiting new AMPAR channels) during learning processes and long-term memory formation (for review see Lisman et al., 2002; Mayford, 2007). Most research has been done in mammals, but CaMKII was also shown to play a role in invertebrate learning and memory (Lent et al., 2007; Matsumoto et al., 2014; for a review in *Drosophila* see Malik and Hodge, 2014). I show in my thesis that CaMKII is highly abundant in the mushroom body and distinctly localized in the subpopulation of noncompact Kenyon cells, an observation that is also supported by the occurrence of RNA in the cell bodies of this neuronal population as shown in previous in-situ hybridizations (Kamikouchi et al., 2000). The immunolocalization studies in my thesis represent an important step towards a functional understanding as they show that CaMKII protein is localized in the dendritic arborizations of the noncompact cells that mainly form two subregions of the calyx, the lip receiving olfactory input (Kirschner et al., 2006) and the collar receiving visual stimuli (Ehmer and Gronenberg, 2002). In both regions CaMKII is found highly concentrated and co-localized with f-actin in the postsynaptic compartments of the microglomeruli, corresponding to the Kenyon cell dendritic spines (Frambach et al., 2004). In the honeybee these spines have been identified to express plastic changes in size and shape correlated to age (Coss et al., 1980) and sensory exposure (Brandon and Coss, 1982). Furthermore, after the transition to foraging the number of spine synapses contacting an individual microglomerulus is increased by ~33% (Groh et al., 2012). These structural changes at the level of individual dendritic spines could be mediated by CaMKII as it may interact directly with the cytoskeleton protein f-actin (Okamoto et al., 2009). It is possible that structural changes of individual spines in turn may affect the reorganization of entire microglomeruli via so far unknown pathways. This could be an explanation for the observed age- and sensory exposure-related pruning of microglomeruli or, following associative learning and long-term memory formation, the emergence of new microglomeruli (Hourcade et al., 2010). To gain a better understanding of the role that CaMKII might play in structural plasticity associated with sensory exposure and learning and

memory processes it would be useful to establish an RNAi mediated knockdown of the protein. Using this approach in combination with different learning paradigms and live-cell imaging will further promote our understanding of molecular mechanism underlying structural neuronal plasticity in the mushroom body calyx and its functional consequences.

Interestingly CaMKII appears to be absent in the outer compact cells (also termed clawed Kenyon cells) arborizing in all calyx subregions and in the inner compact cells which form the basal ring (see also Kamikouchi et al., 2000). In contrast to the lip and collar, the basal ring receives combined input from both (olfactory and visual) modalities (Ehmer and Gronenberg, 2002; Kirschner et al., 2006). The lack of CaMKII in the basal ring may indicate that this region is less plastic compared to the lip and collar. In fact, my measurements show that the volume increase is less pronounced in the basal ring (see also Durst et al., 1994). However, it could also mean that neuronal plasticity is mediated via different molecular mechanisms. In this context future experiments should tackle the question whether only certain “quality aspects” of visual and olfactory information are processed in the basal ring that do not require a high degree or a completely different type of neuronal plasticity. This is also supported by the fact that microglomeruli in the basal ring have a very different shape compared to the lip and collar.

Final conclusions and outlook

Division of labor is based on an age-related polyethism and is associated with plastic changes in higher brain regions. Age, however, just represents a general baseline for behavioral maturation. The actual needs of the colony represent the most crucial fitness factor meaning that individual behavioral maturation of a worker needs to be modifiable by external factors to maintain the appropriate equilibrium of the workforce distribution in the colony. The studies within my thesis contribute a solid framework for the cellular change underlying plasticity during normal maturation. The future challenge still lies in deciphering the pathways and mechanism that contribute to modulation. A major problem for the experimental access of the mechanism that mediate the action of external factors is that they are multiple in nature, not always obvious, and interconnected within a complex network including multiple feedback loops. This makes it rather difficult to isolate individual factors and investigate whether they act upstream or downstream to each other. For example: Ethyl oleate was shown to be the primer pheromone mediating forager presence

and to alter juvenile hormone titers, which then could ultimately delay behavioral maturation. But what is the role of brain in this cascade? Does juvenile hormone inhibit structural plasticity changes or does the brain mainly follow changes dictated by changes in the sensory environment? Which parts of neuronal development can be assigned to “experience-expectant” or “experience-dependent” plasticity? As the studies in my thesis indicate that ethyl oleate is unlikely to act directly on neuronal maturation, it may still act via other pathways, for example the biogenic amine network to finally influence the progress or nature of structural plasticity. Or does ethyl oleate rather enforce basal activity levels, thus leading to changes in sensory exposure that in turn trigger synaptic plasticity in the mushroom bodies? So far, there is no clear answer to these questions, and future studies will need to continue to carefully disentangle the interplay of pheromones, other biotic and abiotic factors, sensory stimuli and internal programs like the hormonal system to better understand the crucial timing of behavioral transitions and the underlying effects at the level of the brain and behavior. For this, further steps in the direction of analyzing the molecular mechanisms underlying structural neuronal plasticity (for example with regards to CaMKII) are most promising approaches as these pathways can be connected to modulating factors for behavioral maturation.

In general, understanding of the mechanisms regulating behavioral maturation and division of labor represents an important step towards understanding social organization in insects and the evolution of this enormously successful life form. Furthermore, these studies provide important insights into fundamental processes of neuronal plasticity mediating behavioral plasticity. The honeybee will be an excellent model organism in the future to pursue these goals.

Bibliography

Abel R, Rybak J, Menzel R. 2001. Structure and response patterns of olfactory interneurons in the honeybee, *Apis mellifera*. J Comp Neurol 437:363–383.

Abraham WC. 2008. Metaplasticity: tuning synapses and networks for plasticity. Nat Neurosci 9:387-399.

Altfelder K, Müller U, Menzel R. 1991. Ca^{2+}/calmodulin and Ca^{2+}/phospholipid-dependant protein kinases in the neural tissue of the honeybee *Apis mellifera*. Insect Biochem 21: 479-486.

Amdam GV, Omholt SW. 2003. The hive bee to forager transition in honeybee colonies: the double repressor hypothesis. J Theor Biol 223:451–464.

Arenas A, Giurfa M, Sandoz J-C, Hourcade B, Devaud J-M, Farina WM. 2012. Early olfactory experience induces structural changes in the primary olfactory center of an insect brain. Eur J Neurosci 35:682-690

Barria A, Muller D, Derkach V, Griffith LC, Soderling TR. 1997. Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. Science 276:2042-2045.

Beggs KT, Glendining KA, Marechal NM, Vergoz V, Nakamura I, Slessor KN, Mercer AR. 2007. Queen pheromone modulates brain dopamine function in worker honey bees. Proc Natl Acad Sci U S A 104:2460–2464.

Beggs KT, Mercer AR. 2009. Dopamine receptor activation by honey bee queen pheromone. Curr Biol 19:1206–1209.

Behrends A, Scheiner R. 2009. Evidence for associative learning in newly emerged honey bees (*Apis mellifera*). Anim Cogn 12:249-255.

Beshers SN, Fewell JH. 2001. Models of division of labor in social insects. Annu Rev Entomol 46:413–440.

Bitterman ME, Menzel R, Fietz A, Schäfer S (1983) Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). J Comp Psychol 97:107-119.

Blenau W, Schmidt M, Faensen D, Schürmann F-W. 1999. Neurons with dopamine-like immunoreactivity target mushroom body Kenyon cell somata in the brain of some hymenopteran insects. Int J Insect Morphol Embryol 28:203–210.

Bloch G, Sullivan JP, Robinson GE. 2002. Juvenile hormone and circadian locomotor activity in the honey bee *Apis mellifera*. J Insect Physiol 48:1123–1131.

Brandon JG, Coss RG. 1982. Rapid dendritic spine stem shortening during one-trial learning: the honeybee's first orientation flight. Brain Res 252:51–61.

Brandstaetter AS, Rössler W, Kleineidam CJ (2011) Friends and foes from an ant brain's point of view – neuronal correlates of colony odorsin a social insect. PLoS One 6:e21383.

Brill MF, Rosenbaum T, Reus I, Kleineidam CJ, Nawrot MP, Rössler W. 2013. Parallel processing via a dual olfactory pathway in the honeybee. J Neurosci 33:2443–56.

Brillet C, Robinson GE, Bues R, Le Conte Y. 2002. Racial Differences in Division of Labor in Colonies of the Honey Bee (*Apis mellifera*). Ethology 108:115–126.

Brown SM, Napper RM, Mercer AR. 2004. Foraging experience, glomerulus volume, and synapse number: A stereological study of the honey bee antennal lobe. J Neurobiol 60:40–50.

Brown SM, Napper RM, Thompson CM, Mercer AR. 2002. Stereological analysis reveals striking differences in the structural plasticity of two readily identifiable glomeruli in the antennal lobes of the adult worker honeybee. J Neurosci 22:8514–8522.

Burkert P, Duch C. 2006. Developmental changes of CaMKII localization, activity and function during postembryonic CNS remodelling in *Manduca sexta*. Eur J Neurosci 23:335-349.

Butler C. 1609. The feminine monarchie. Oxford, Joseph Barnes

Capaldi EA, Smith AD, Osborne JL, Fahrbach SE, Farris SM, Reynolds DR, Edwards AS, Martin A, Robinson GE, Poppy GM, Riley JR. 2000. Ontogeny of orientation flight in the honeybee revealed by harmonic radar. Nature 403:537–540.

Castillo C, Chen H, Graves C, Maisonnasse A, Le Conte Y, Plettner E. 2012a. Biosynthesis of ethyl oleate, a primer pheromone, in the honey bee (*Apis mellifera* L.). Insect Biochem Mol Biol 42:404–416.

Castillo C, Maisonnasse A, Le Conte Y, Plettner E. 2012b. Seasonal variation in the titers and biosynthesis of the primer pheromone ethyl oleate in honey bees. J Insect Physiol 58:1112–1121.

Chen H-X, Otmakhov N, Strack S, Colbran RJ, Lisman JE. 2001. Is persistent activity of calcium/calmodulin-dependent kinase required for the maintenance of LTP? J Neurophysiol 85:1368-1376.

Cho K-O, Wall JB, Pugh PC, Ito M, Mueller SA, Kennedy MB. 1991. The alpha subunit of type II Ca^{2+}/calmodulin-dependent protein kinase is highly conserved in *Drosophila*. Neuron 7:439-450.

Le Conte Y, Arnold G, Trouiller J, Masson C, Chappe B. 1990. Identification of a brood pheromone in honeybees. Naturwissenschaften 77:334–336.

Le Conte Y, Bécard J-M, Costagliola G, de Vaublanc G, El Maâtaoui M, Crauser D, Plettner E, Slessor KN. 2006. Larval salivary glands are a source of primer and releaser pheromone in honey bee (*Apis mellifera* L.). Naturwissenschaften 93:237–241.

Le Conte Y, Hefetz A. 2008. Primer pheromones in social hymenoptera. Annu Rev Entomol 53:523–542.

Le Conte Y, Mohammedi A, Robinson GE. 2001. Primer effects of a brood pheromone on honeybee behavioural development. Proc Biol Sci 268:163–168.

Coss RG, Brandon JG, Globus A. 1980. Changes in morphology of dendritic spines on honeybee calycal interneurons associated with cumulative nursing and foraging experiences. Brain Res 192:49–59.

Davis RL. 2005. Olfactory memory formation in *Drosophila*: from molecular to systems neuroscience. Annu Rev Neurosci 28:275-302.

Dobrin SE, Fahrbach SE. 2012. Rho GTPase activity in the honey bee mushroom bodies is correlated with age and foraging experience. J Insect Physiol 58:228–234.

Dobrin SE, Herlihy JD, Robinson GE, Fahrbach SE. 2011. Muscarinic regulation of Kenyon cell dendritic arborizations in adult worker honey bees. Arthropod Struct Dev 40:409–419.

Dujardin F. 1850. Mémoire sur le système nerveux des insectes. Ann Sci Natl Zool:195–205.

Durst C, Eichmüller S, Menzel R. 1994. Development and experience lead to increased volume of subcompartments of the honeybee mushroom body. Behav Neural Biol 62:259–263.

Ehmer B, Gronenberg W. 2002. Segregation of visual input to the mushroom bodies in the honeybee (*Apis mellifera*). J Comp Neurol 451:362–373.

Eichmüller S. 1994. Vom Sensillum zum Pilzkörper: Immunhistologische und ontogenetische Aspekte zur Anatomie des olfaktorischen Systems der Honigbiene. Dissertation. Freie Universität Berlin.

Engels W, Fahrenhorst H. 1974. Alters- und kastenspezifische Veränderungen der Haemolymph-Protein-Spektren bei *Apis mellificia*. Wilhelm Roux Arch Entwickl Mech Org 174:285–296.

Erber J, Masuhr T, Menzel R. 1980. Localization of short-term memory in the brain of the bee, *Apis mellifera*. Physiol Entomol 5:343-358.

Erondu NE, Kennedy MB. 1985. Regional distribution of type II Ca^{2+}/calmodulin-dependent protein kinase in rat brain. J Neurosci 5:3270-3277.

Fahrbach SE, Dobrin SE. 2008. The How and Why of Structural Plasticity in the Adult Honeybee Brain. In: Dukas R, Ratcliffe JM, editors. Cognitive Ecology II. University of Chicago Press. p 27–46.

Fahrbach SE, Farris SM, Sullivan JP, Robinson GE. 2003. Limits on volume changes in the mushroom bodies of the honey bee brain. J Neurobiol 57:141–151.

Fahrbach SE, Moore D, Capaldi EA, Farris SM, Robinson GE. 1998. Experience-expectant plasticity in the mushroom bodies of the honeybee. Learn Mem 5:115–123.

Fahrbach SE, Robinson GE. 1996. Juvenile hormone, behavioral maturation, and brain structure in the honey bee. Dev Neurosci 18:102–114.

Fahrbach SE, Strande JL, Robinson GE. 1995. Neurogenesis is absent in the brains of adult honey bees and does not explain behavioral neuroplasticity. Neurosci Lett 197:145–8.

Fahrbach SE. 2006. Structure of the mushroom bodies of the insect brain. Annu Rev Entomol 51:209–232.

Farris SM. 2005. Evolution of insect mushroom bodies: old clues, new insights. Arthropod Struct Dev 34:211–234.

Farris SM, Davis RL, Robinson GE, Fahrbach SE. 1999. Larval and pupal development of the mushroom bodies in the honey bee, *Apis mellifera*. J Comp Neurol 414:97-113.

Farris SM, Fahrbach SE, Robinson GE. 2001. Experience- and age-related outgrowth of intrinsic neurons in the mushroom bodies of the adult worker honeybee. J Neurosci 21:6395–6404.

Farris SM, Sinakevitch IT. 2003. Development and evolution of the insect mushroom bodies: towards the understanding of conserved developmental mechanisms in a higher brain center. Arthropod Struct Dev 32:79–101.

Farris SM, Strausfeld NJ, Abrams AI. 2004. Development and morphology of class II Kenyon cells in the mushroom bodies of the honey bee, *Apis mellifera*. J Comp Neurol 474:325-339.

Fox K. 2003. Synaptic plasticity: the subcellular location of CaMKII controls plasticity. Curr Biol 13:R143-R145.

Frambach I, Rössler W, Winkler M, Schürmann F-W. 2004. F-actin at identified synapses in the mushroom body neuropil of the insect brain. J Comp Neurol 475:303–314.

Free JB. 1965. The allocation of duties among worker honeybees. Symp Zool Soc London 14:39-59.

Von Frisch K. 1967. The dance language and orientation of honeybees. Cambridge, Massachusetts, USA: Havard University Press.

Galizia CG, Rössler W. 2010. Parallel olfactory systems in insects: anatomy and function. Annu Rev Entomol 55:399-420.

Ganeshina O, Menzel R. 2001. GABA-immunoreactive neurons in the mushroom bodies of the honeybee: an electron microscopic study. J Comp Neurol 437:335–349.

Ganeshina O, Schäfer S, Malun D. 2000. Proliferation and programmed cell death of neuronal precursors in the mushroom bodies of the honeybee. J Comp Neurol 417:349–365.

Gerber B, Tanimoto H, Heisenberg M. 2004. An engram found? Evaluating the evidence from fruit flies. Curr Opin Neurobiol 14:737-744.

Giray T, Robinson GE. 1994. Effects of intracolony variability in behavioral development on plasticity of division of labor in honey bee colonies. Behav Ecol Sociobiol 35:13–20.

Giurfa M. 2007. Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. J Comp Physiol A 193:801–824.

Giurfa M, Sandoz J-C. 2012. Invertebrate learning and memory: Fifty years of olfactory conditioning of the proboscis extension response in honeybees. Learn Mem 19:54–66.

Godenschwege TA, Reisch D, Diegelmann S, Eberle K, Funk N, Heisenberg M, Hoppe V, Hoppe J, Klagges BRE, Martin J-R, et al. 2004. Flies lacking all synapsins are unexpectedly healthy but are impaired in complex behaviour. Eur J Neurosci 20:611-622.

Griffith LC, Greenspan RJ. 1993. The diversity of calcium/calmodulin-dependent protein kinase II isoforms in *Drosophila* is generated by alternative splicing of a single gene. J Neurochem 61:1534-1537.

Griffith LC, Lu CS, Sun XX. 2003. CaMKII, an enzyme on the move: regulation of temporospatial localization. Mol Interv 3:386-403.

Griffith LC. 2004. Calcium/calmodulin-dependent protein kinase II: an unforgettable kinase. J Neurosci 24:8391-8393.

Groh C, Ahrens D, Rössler W. 2006. Environment- and age-dependent plasticity of synaptic complexes in the mushroom bodies of honeybee queens. Brain Behav Evol 68:1–14.

Groh C, Rössler W. 2008. Caste-specific postembryonic development of primary and secondary olfactory centers in the female honeybee brain. Arthropod Struct Dev 37:459-468.

Groh C, Rössler W. 2011. Comparison of microglomerular structures in the mushroom body calyx of neopteran insects. Arthropod Struct Dev, 40:358-367.

Groh C, Lu Z, Meinertzhagen IA, Rössler W. 2012. Age-related plasticity in the synaptic ultrastructure of neurons in the mushroom body calyx of the adult honeybee *Apis mellifera*. J Comp Neurol 520:3509–3527.

Groh C, Tautz J, Rössler W. 2004. Synaptic organization in the adult honey bee brain is influenced by brood-temperature control during pupal development. Proc Natl Acad Sci USA 101:4268–4273.

Gronenberg W, Heeren S, Hölldobler B. 1996. Age-dependent and task-related morphological changes in the brain and the mushroom bodies of the ant *Camponotus floridanus*. J Exp Biol 199:2011–2019.

Gronenberg W. 2001. Subdivisions of hymenopteran mushroom body calyces by their afferent supply. J Comp Neurol 435:474–489.

Grünewald B. 1999. Morphology of feedback neurons in the mushroom body of the honeybee, *Apis mellifera*. J Comp Neurol 404:114–126.

Guidugli KR, Amdam G V., Nascimento AM, Barchuk AR, Omholt S, Simões ZLP, Hartfelder K. 2005. Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect. FEBS Lett 579:4961–4965.

Hagenguth H, Rembold H. 1978. Identification of juvenile hormone 3 as the only JH homolog in all developmental stages of the honey bee. Z Naturforsch:847–850.

Hammer M. 1993. An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. Nature 366:59–63.

Hammer M, Menzel R. 1998. Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. Learn Mem 5:146–56.

Heisenberg M. 1998. What do the mushroom bodies do for the insect brain? An introduction. Learn Memory 5:1-10.

Heisenberg M. 2003. Mushroom body memoir: from maps to models. Nat Neurosci 4:266-275.

Hölldobler B, Wilson EO. 2008. The Superorganism: The Beauty, Elegance, and Strangeness of Insect Societies. 1. Edition. W. W. Norton & Company.

Hofbauer A, Ebel T, Waltenspiel B, Oswald P, Chen YC, Halder P, Biskup S, Lewandrowski U, Winkler C, Sickmann A, Buchner S, Buchner E. 2009 The wuerzburg hybridoma library against *Drosophila* brain. J Neurogenet 23:78-91.

Hoover SER, Keeling CI, Winston ML, Slessor KN. 2003. The effect of queen pheromones on worker honey bee ovary development. Naturwissenschaften 90:477–480.

Hourcade B, Muenz TS, Sandoz J-C, Rössler W, Devaud J-M. 2010. Long-term memory leads to synaptic reorganization in the mushroom bodies: a memory trace in the insect brain? J Neurosci 30:6461–6465.

Hourcade B, Perisse E, Devaud J-M, Sandoz J-C. 2009. Long-term memory shapes the primary olfactory center of an insect brain. Learn Mem 16:607–615.

Huang Z-Y, Plettner E, Robinson GE. 1998. Effects of social environment and worker mandibular glands on endocrine-mediated behavioral development in honey bees. J Comp Physiol A 183:143–152.

Huang Z-Y, Robinson GE. 1992. Honeybee colony integration: worker-worker interactions mediate hormonally regulated plasticity in division of labor. Proc Natl Acad Sci USA 89:11726-11729.

Huang Z-Y, Robinson GE. 1996. Regulation of honey bee division of labor by colony age demography. Behav Ecol Sociobiol 39:147-158.

Hughes WOH, Boomsma JJ. 2007. Genetic polymorphism in leaf-cutting ants is phenotypically plastic. Proc Biol Sci 274:1625–1630.

Irvine EE, Vernon J, Giese KP. 2005. AlphaCaMKII autophosphorylation contributes to rapid learning but is not necessary for memory. Nat Neurosci 8:411-412.

Ismail N, Fahrbach SE, Robinson GE. 2006. Stimulation of muscarinic receptors mimics experience-dependent plasticity in the honey bee brain. Proc Natl Acad Sci USA 103:207–211.

Ito K, Shinomiya K, Ito M, Armstrong JD, Boyan G, Hartenstein V, Harzsch S, Heisenberg M, Homberg U, Jenett A, Keshishian H, Restifo LL, Rössler W, Simpson JH, Strausfeld NJ, Strauss R, Vosshall LB. 2014. A systematic nomenclature for the insect brain. Neuron 81:755–765.

Johnson BR. 2010. Division of labor in honeybees: form, function, and proximate mechanisms. Behav Ecol Sociobiol 64:305–316.

Jones TA, Donlan NA, O'Donnell S. 2009. Growth and pruning of mushroom body Kenyon cell dendrites during worker behavioral development in the paper wasp, *Polybia aequatorialis* (Hymenoptera: Vespidae). Neurobiol Learn Mem 92:485–495.

Kaatz H, Hildebrandt H, Engels W. 1992. Primer effect of queen pheromone on juvenile hormone biosynthesis in adult worker honey bees. J Comp Physiol B 162:588–592.

Kaech S, Parmar H, Roelandse M, Bornmann C, Matus A. 2001. Cytoskeletal microdifferentiation: a mechanism for organizing morphological plasticity in dendrites. Proc Natl Acad Sci USA 98:7086–7092.

Kamikouchi A, Takeuchi H, Sawata M, Natori S, Kubo T. 2000. Concentrated expression of Ca^{2+}/ calmodulin-dependent protein kinase II and protein kinase C in the mushroom bodies of the brain of the honeybee *Apis mellifera* L. J Comp Neurol 417:501–510.

Keeling CI, Slessor KN. 2005. A scientific note on aliphatic esters in queen honey bees. Apidologie 36: 559-560.

Keeling CI, Slessor KN, Higo HA, Winston ML. 2003. New components of the honey bee (*Apis mellifera* L.) queen retinue pheromone. Proc Natl Acad Sci USA 100:4486–91.

Kelleher RJ, Govindarajan A, Tonegawa S. 2004. Translational regulatory mechanisms in persistent forms of synaptic plasticity. Neuron 44:59-73.

Kennedy MB, Bennett MK, Erondu NE. 1983. Biochemical and immunochemical evidence that the "major postsynaptic density protein" is a subunit of a calmodulin-dependent protein kinase. Proc Natl Acad Sci USA 80:7357-7361.

Kenyon F. 1896. The brain of the bee. A preliminary contribution to the morphology of the nervous system of the arthropoda. J Comp Neurol 6:133–210.

Kirschner S, Kleineidam CJ, Zube C, Rybak J, Grünewald B, Rössler W. 2006. Dual olfactory pathway in the honeybee, *Apis mellifera*. J Comp Neurol 499:933–952.

Klagges BRE, Heimbeck G, Godenschwege TA, Hofbauer A, Pflugfelder GO, Reifegerste R, Reisch D, Schaupp M, Buchner S, Buchner E. 1996. Invertebrate synapsins: A single gene codes for several isoforms in *Drosophila*. J Neurosci 16:3154-3165.

Kojundzic SL, Puljak L, Hogan Q, Sapunar D. 2010. Depression of Ca^{2+} /calmodulin-dependent protein kinase II in dorsal root ganglion neurons after spinal nerve ligation. J Comp Neurol 518:64-74.

Kreissl S, Eichmüller S, Bicker G, Rapus J, Eckert M. 1994. Octopamine-like immunoreactivity in the brain and subesophageal ganglion of the honeybee. J Comp Neurol 348:583–595.

Kremer MC, Christiansen F, Leiss F, Paehler M, Knapek S, Andlauer TFM, Förstner F, Kloppenburg P, Sigrist SJ, Tavosanis G. 2010. Structural Long-Term Changes at Mushroom Body Input Synapses. Curr Biol 20:1938-1944.

Krofczik S, Khojasteh U, Hempel de Ibarra N, Menzel R. 2008. Adaptation of microglomerular complexes in the honeybee mushroom body lip to manipulations of behavioral maturation and sensory experience. Dev Neurobiol 68:1007–1017.

Kucharski R, Maleszka J, Foret S, Maleszka R. 2008. Nutritional control of reproductive status in honeybees via DNA methylation. Science 319:1827–1830.

Kühn-Bühlmann S, Wehner R. 2006. Age-dependent and task-related volume changes in the mushroom bodies of visually guided desert ants, *Cataglyphis bicolor*. J Neurobiol 66:511–521.

Ledoux MN, Winston ML, Higo H, Keeling CI, Slessor KN, LeConte Y. 2001. Queen and pheromonal factors influencing comb construction by simulated honey bee (*Apis mellifera* L.) swarms. Insectes Soc 48:14–20.

Leiss F, Groh C, Butcher NJ, Meinertzhagen IA, Tavosanis G. 2009. Synaptic organization in the adult *Drosophila* mushroom body calyx. J Comp Neurol 517:808-824.

Lent DD, Pintér M, Strausfeld NJ. 2007. Learning with half a brain. Dev Neurobiol 67:740–751.

Leoncini I, Le Conte Y, Costagliola G, Toth AL, Plettner E, Wang M, Huang Z, Bécard J-M, Slessor KN, Crauser D, Robinson GE. 2004a. Regulation of behavioral maturation by a primer pheromone produced by adult worker honey bees. Proc Natl Acad Sci USA 101:17559–17564.

Leoncini I, Crauser D, Robinson GE, Le Conte Y. 2004b. Worker-worker inhibition of honey bee behavioural development independent of queen and. Insectes Soc 51:392–394.

Lindauer M. 1952. Ein Beitrag zur Frage der Arbeitsteilung im Bienenstaat. Z Vgl Physiol 34:299–345.

Lindauer M. 1961. Communication among social bees. Havard University Press, Cambridge, MA.

Lisman JE, Goldring MA. 1988. Feasibility of long-term storage of graded information by the Ca^{2+}/calmodulin-dependent protein kinase molecules of the postsynaptic density. Proc Natl Acad Sci USA 85:5320-5324.

Lisman JE, Schulman H, Cline H. 2002. The molecular basis of CaMKII function in synaptic and behavioural memory. Nat Rev Neurosci 3:175–190.

Lisman JE. 1989. A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory. Proc Natl Acad Sci USA 86:9574-9578.

Locatelli F, Bundrock G, Müller U. 2005. Focal and temporal release of glutamate in the mushroom bodies improves olfactory memory in *Apis mellifera*. J Neurosci 25:11614–11618.

Lozano V, Armengaud C, Gauthier M. 2001. Memory impairment induced by cholinergic antagonists injected into the mushroom bodies of the honeybee. J Comp Physiol A 187:249–254.

Lou LL, Lloyd SJ, Schulman H. 1986. Activation of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation: ATP modulates production of an autonomous enzyme. Proc Natl Acad Sci USA 83:9497-9501.

Malenka RC, Kauer JA, Perkel DJ, Mauk MD, Kelly PT, Nicoll RA, Waxham MN. 1989. An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. Nature 340:554–557.

Malik BR, Hodge JJL. 2014. CASK and CaMKII function in *Drosophila* memory. Front Neurosci 8:178.

Matsumoto Y, Sandoz J-C, Devaud J-M, Lormant F, Mizunami M, Giurfa M. 2014. Cyclic nucleotide-gated channels, calmodulin, adenylyl cyclase, and calcium/calmodulin-dependent protein kinase II are required for late, but not early, long-term memory formation in the honeybee. Learn Mem 21:272–286.

Mayford M. 2007. Protein kinase signaling in synaptic plasticity and memory. Curr Opin Neurobiol 17:313–317.

Menzel R. 1999. Memory dynamics in the honeybee. J Comp Physiol A S 185:323–340.

Menzel R. 2001. Searching for the memory trace in a mini-brain, the honeybee. Learn Mem 8:53–62.

Menzel R, Giurfa M. 2001. Cognitive architecture of a mini-brain: the honeybee. Trends Cogn Sci 5:62–71.

Menzel R, Giurfa M. 2006. Dimensions of cognition in an insect, the honeybee. Behav Cogn Neurosci Rev 5:24-40.

Miller SG, Kennedy MB. 1986. Regulation of brain type II Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation: a Ca^{2+}-triggered molecular switch. Cell 44:861-870.

Mobbs PG. 1982. The Brain of the Honeybee *Apis Mellifera*. I. The Connections and Spatial Organization of the Mushroom Bodies. Philos Trans R Soc B Biol Sci 298:309–354.

Mohammedi A, Crauser D. 1996. Effect of a brood pheromone on honeybee hypopharyngeal glands. Comptes Rendus de L'Academie des Sciences. Serie III, Sciences de la vie 319:769-772.

Mohammedi A, Paris A, Crauser D, Le Conte Y. 1998. Effect of Aliphatic Esters on Ovary Development of Queenless Bees (*Apis mellifera* L.). Naturwissenschaften 85:455–458.

Morgan SM, Butz Huryn VM, Downes SR, Mercer AR. 1998. The effects of queenlessness on the maturation of the honey bee olfactory system. Behav Brain Res 91:115–126.

Muenz TS, Maisonnasse A, Plettner E, Le Conte Y, Rössler W. 2012. Sensory reception of the primer pheromone ethyl oleate. Naturwissenschaften 99:421–425.

Muenz TS, Oberwallner B, Gehring K, Rössler W. 2008. Plasticity of synaptic complexes in the mushroom bodies of the honeybee brain depends on age, experience and season. In: FENS Abstr 4:143.

Nelson CM, Ihle KE, Fondrk MK, Page RE, Amdam G V. 2007. The gene vitellogenin has multiple coordinating effects on social organization. PLoS Biol 5:e62.

O'Donnell S, Donlan NA, Jones TA. 2004. Mushroom body structural change is associated with division of labor in eusocial wasp workers (*Polybia aequatorialis*, Hymenoptera: Vespidae). Neurosci Lett 356:159–162.

Ohsako S, Nishida Y, Ryo H, Yamauchi T. 1993. Molecular characterization and expression of the *Drosophila* Ca^{2+}/calmodulin-dependent protein kinase II gene. Identification of four forms of the enzyme generated from a single gene by alternative splicing. J Biol Chem 268:2052-2062.

Okamoto K, Bosch M, Hayashi Y. 2009. The roles of CaMKII and F-actin in the structural plasticity of dendritic spines: a potential molecular identity of a synaptic tag? Physiology 24:357-366.

Page R, Pankiw T. 2001. Genotype and colony environment affect honeybee (*Apis mellifera* L.) development and foraging behavior. Behav Ecol Sociobiol 51:87–94.

Page RE, Scheiner R, Amdam G V., Erber J. 2006. The development and evolution of division of labor and foraging specialization in a social insect (*Apis mellifera* L.). Curr Top Dev Biol 74:253–286.

Pankiw T, Huang Z-Y, Winston ML, Robinson GE. 1998a. Queen mandibular gland pheromone influences worker honey bee (*Apis mellifera* L.) foraging ontogeny and juvenile hormone titers. J Insect Physiol 44:685–692.

Pankiw T, Page RE, Fondrk MK. 1998b. Brood pheromone stimulates pollen foraging in honey bees (*Apis mellifera*). Behav Ecol Sociobiol 44:193–198.

Pankiw T, Page RE. 1999. The effect of genotype, age, sex, and caste on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). J Comp Physiol A 185:207–213.

Pankiw T, Page RE. 2003. Effect of pheromones, hormones, and handling on sucrose response thresholds of honey bees (*Apis mellifera* L.). J Comp Physiol A 189:675–684.

Pankiw T. 2004. Worker honey bee pheromone regulation of foraging ontogeny. Naturwissenschaften 91:178–181.

Pasch E, Muenz TS, Rössler W. 2011. CaMKII is differentially localized in synaptic regions of Kenyon cells within the mushroom bodies of the honeybee brain. J Comp Neurol 519:3700–3712.

Paulk AC, Dacks AM, Phillips-Portillo J, Fellous J-M, Gronenberg W. 2009. Visual processing in the central bee brain. J Neurosci 29:9987–9999.

Paulk AC, Gronenberg W. 2008. Higher order visual input to the mushroom bodies in the bee, *Bombus impatiens*. Arthropod Struct Dev 37:443–458.

Perisse E, Raymond-Delpech V, Néant I, Matsumoto Y, Leclerc C, Moreau M, Sandoz J-C. 2009. Early calcium increase triggers the formation of olfactory long-term memory in honeybees. BMC Biol 7:30.

Pinto LZ, Bitondi MM., Simões ZL. 2000. Inhibition of vitellogenin synthesis in *Apis mellifera* workers by a juvenile hormone analogue, pyriproxyfen. J Insect Physiol 46:153–160.

Ramón y Cajal S. 1891. Sur la structure de l'écorce cérébrale de quelques mammifères. La Cell 7.

Reiner DJ, Newton EM, Tian H, Thomas JH. 1999. Diverse behavioural defects caused by mutations in *Caenorhabditis elegans* unc-43 CaM kinase II. Nature 402:199-203.

Robinson GE, Huang Z-Y. 1998. Colony integration in honey bees: genetic, endocrine and social control of division of labor. Apidologie 29:159–170.

Robinson GE, Page RE, Strambi C, Strambi A. 1989. Hormonal and Genetic Control of Behavioral Integration in Honey Bee Colonies. Science 246:109–112.

Robinson GE. 1992. Regulation of division of labor in insect societies. Annu Rev Entomol 37:637–665.

Rosenberg OS, Deindl S, Sung R-J, Nairn AC, Kuriyan J. 2005. Structure of the autoinhibited kinase domain of CaMKII and SAXS analysis of the holoenzyme. Cell 123:849-860.

Rösch G. 1925. Untersuchungen über dieArbeitsteilung im Bienenstaat. 1. Teil: Die Tätigkeiten im normalen Bienenstaate und ihre Beziehungen zum Alter der Arbeitsbienen. Z Vgl Physiol 2:571–631.

Rösch G. 1930. Untersuchungen über die Arbeitsteilung im Bienenstaat. 2. Teil: Die Tätigkeiten der Arbeistbienen unter experimentell veränderten Bedingungen. Z Vgl Physiol 12:1–71.

Rössler W, Brill MF. 2013. Parallel processing in the honeybee olfactory pathway: structure, function, and evolution. J Comp Physiol A 199:981-996

Rössler W, Groh C. 2012. Plasticity of synaptic microcircuits in the mushroom-body calyx of the honey bee. In: Honeybee Neurobiology and Behaviour. Springer. p 141–151.

Rybak J, Menzel R. 1993. Anatomy of the mushroom bodies in the honey bee brain: the neuronal connections of the alpha-lobe. J Comp Neurol 334:444–465.

Scheiner R, Baumann A, Blenau W. 2006. Aminergic control and modulation of honeybee behaviour. Curr Neuropharmacol 4:259–276.

Scholl C, Wang Y, Krischke M, Mueller MJ, Amdam G V, Rössler W. 2014. Light exposure leads to reorganization of microglomeruli in the mushroom bodies and influences juvenile hormone levels in the honeybee. Dev Neurobiol 74:1141–1153.

Schröter U, Menzel R. 2003. A new ascending sensory tract to the calyces of the honeybee mushroom body, the subesophageal-calycal tract. J Comp Neurol 465:168–178.

Schulz DJ, Barron AB, Robinson GE. 2002. A role for octopamine in honey bee division of labor. Brain Behav Evol 60:350–359.

Schulz DJ, Robinson GE. 1999. Biogenic amines and division of labor in honey bee colonies: behaviorally related changes in the antennal lobes and age-related changes in the mushroom bodies. J Comp Physiol A 184:481–488.

Schulz DJ, Robinson GE. 2001. Octopamine influences division of labor in honey bee colonies. J Comp Physiol A 187:53–61.

Schworer CM, Colbran RJ, Soderling TR. 1986. Reversible generation of a Ca^{2+}-independent form of Ca^{2+}(calmodulin)-dependent protein kinase II by an autophosphorylation mechanism. J Biol Chem 261:8581-8584.

Seeley TD. 1982. Adaptive significance of the age polyethism schedule in honeybee colonies. Behav Ecol and Sociobiol 11:287-293.

Seeley TD. 1985. Honeybee Ecology: A Study of Adaptation in Social Life (Monographs in Behavior & Ecology). Princeton Univ Pr., Princeton

Seid MA, Harris KM, Traniello JFA. 2005. Age-related changes in the number and structure of synapses in the lip region of the mushroom bodies in the ant Pheidole dentata. J Comp Neurol 488:269–277.

Seid MA, Wehner R. 2009. Delayed axonal pruning in the ant brain: a study of developmental trajectories. Dev Neurobiol 69:350–364.

Shanavas A, Dutta-Gupta A, Murthy CRK. 1998. Identification, characterization, immunocytochemical localization, and developmental changes in the activity of calcium/calmodulin-dependent protein kinase II in the CNS of *Bombyx mori* during postembryonic development. J Neurochem 70:1644-1651.

Sigg D, Thompson CM, Mercer AR. 1997. Activity-dependent changes to the brain and behavior of the honey bee, *Apis mellifera* (L.). J Neurosci 17:7148-7156.

Silva AJ, Paylor R, Wehner JM, Tonegawa S. 1992a. Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science 257:206-211.

Silva AJ, Stevens CF, Tonegawa S, Wang Y. 1992b. Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. Science 257:201-206.

Slessor KN, Kaminski L-A, King GGS, Borden JH, Winston ML. 1988. Semiochemical basis of the retinue response to queen honey bees. Nature 332:354–356.

Slessor KN, Winston ML, Le Conte Y. 2005. Pheromone communication in the honeybee (*Apis mellifera* L.). J Chem Ecol 31:2731–2745.

Soderling TR. 1993. Calcium/calmodulin-dependent protein kinase II: role in learning and memory. Mol Cell Biochem 127-128:93-101.

Steiger U. 1967. Über den Feinbau des Neuropils im Corpus pedunculatum der Waldameise. Zeitschrift für Zellforsch und Mikroskopische Anat 81:511–536.

Stieb SM, Hellwig A, Wehner R, Rössler W. 2012. Visual experience affects both behavioral and neuronal aspects in the individual life history of the desert ant *Cataglyphis fortis*. Dev Neurobiol 72:729–742.

Stieb SM, Muenz TS, Wehner R, Rössler W. 2010. Visual experience and age affect synaptic organization in the mushroom bodies of the desert ant *Cataglyphis fortis*. Dev Neurobiol 70:408–423.

Strausfeld NJ, Hansen L, Li Y, Gomez RS, Ito K. 1998. Evolution, discovery, and interpretations of arthropod mushroom bodies. Learn Memory 5:11-37.

Strausfeld NJ. 2002. Organization of the honey bee mushroom body: representation of the calyx within the vertical and gamma lobes. J Comp Neurol 450:4–33.

Sullivan JP, Fahrbach SE, Robinson GE. 2000. Juvenile hormone paces behavioral development in the adult worker honey bee. Horm Behav 37:1–14.

Szyszka P, Galkin A, Menzel R. 2008. Associative and non-associative plasticity in Kenyon cells of the honeybee mushroom body. Front Syst Neurosci 2:3.

Takamatsu Y, Kishimoto Y, Ohsako S. 2003. Immunohistochemical study of Ca^{2+}/calmodulin-dependent protein kinase II in the *Drosophila* brain using a specific monoclonal antibody. Brain Res 974:99-116.

The Honeybee Genome Sequencing Consortium. 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443:931-949.

Tobe SS, Stay B. 1985. Structure and Regulation of the Corpus Allatum. Adv In Insect Phys 18:305–432.

Tombes RM, Faison MO, Turbeville JM. 2003. Organization and evolution of multifunctional Ca^{2+}/CaM-dependent protein kinase genes. Gene 322:17-31.

Trujillo-Cenóz O, Melamed J. 1962. Electron microscope observations on the calyces of the insect brain. J Ultrastruct Res 7:389–398.

Vergoz V, Schreurs HA, Mercer AR. 2007. Queen pheromone blocks aversive learning in young worker bees. Science 317:384–386.

Wagener-Hulme C, Kuehn JC, Schulz DJ, Robinson GE. 1999. Biogenic amines and division of labor in honey bee colonies. J Comp Physiol A 184:471-479.

Winnington AP, Napper RM, Mercer AR. 1996. Structural plasticity of identified glomeruli in the antennal lobes of the adult worker honey bee. J Comp Neurol 365:479–490.

Winston ML, Higo HA, Colley SJ, Pankiw T, Slessor KN. 1991. The role of queen mandibular pheromone and colony congestion in honey bee (*Apis mellifera* L.) reproductive swarming (Hymenoptera: Apidae). J Insect Behav 4:649–660.

Winston ML. 1987. The biology of the honey bee. Cambridge, Massachusetts, USA: Havard University Press.

Withers GS, Day NF, Talbot EF, Dobson HEM, Wallace CS. 2008. Experience-dependent plasticity in the mushroom bodies of the solitary bee *Osmia lignaria* (Megachilidae). Dev Neurobiol 68:73–82.

Withers GS, Fahrbach SE, Robinson GE. 1993. Selective neuroanatomical plasticity and division of labour in the honeybee. Nature 364:238–240.

Withers GS, Fahrbach SE, Robinson GE. 1995. Effects of experience and juvenile hormone on the organization of the mushroom bodies of honey bees. J Neurobiol 26:130–144.

Witthöft W. 1967. Absolute Anzahl und Verteilung der Zellen im Hirn der Honigbiene. Zeitschrift für Morphol der Tiere 61:160–184.

Wright GA, Mustard JA, Simcock NK, Ross-Taylor AAR, McNicholas LD, Popescu A, Marion-Poll F. 2010. Parallel reinforcement pathways for conditioned food aversions in the honeybee. Curr Biol 20:2234–2240.

Yasuyama K, Meinertzhagen IA, Schürmann F-W. 2002. Synaptic organization of the mushroom body calyx in *Drosophila melanogaster*. J Comp Neurol 445:211-226.

Yuste R, Denk W. 1995. Dendritic spines as basic functional units of neuronal integration. Nature 375:682–684.

Zars T. 2000. Behavioral functions of the insect mushroom bodies. Curr Opin Neurobiol 10:790-795.

Curriculum Vitae

Thomas Sebastian Münz
Dipl. Biol.

Current job address*:*
Department of Behavioral Physiology and Sociobiology, Zoology II
Biozentrum, University of Würzburg
97074 Würzburg, Germany
Telefon: +49 931 3184313
E-Mail: thomas.muenz@biozentrum.uni-wuerzburg.de

Scientific Education

since Feb 2008 — **PhD thesis: "**Aspects of neuronal plasticity in the mushroom body calyx during adult maturation in the honeybee *Apis mellifera*"
Zoology II, University of Würzburg (Germany)
Supervisor: Prof. Wolfgang Rössler, University of Würzburg (Germany)
Prof. Alison Mercer, University of Otago (New Zealand)

Sep 2006 – Jul 2007 — **Diploma thesis:** "Quantification of synaptic complexes in the mushroom-body calyx of hymenopteran brains"
Zoology II, University of Würzburg (Germany)
Supervisor: Prof. Wolfgang Rössler

Sep 2001 – Jun 2006 — **Biology studies (Diploma)**
University of Würzburg (Germany)
major subject: Behavioral Physiology and Sociobiology
minor subject: Animal ecology and tropical biology
minor subject: Ecophysiology of plants & Vegetation Ecology

Sep 1992 – Jun 2001 — **Abitur (German University entrance qualifikation)**
staatl. Gymnasium mit Schülerheim Hohenschwangau (Germany)

Research Internships

Feb 2010 – Mar 2010 — **Research stay**
Prof. Alison Mercer, Department of Zoology, University of Otago (New Zealand)

Jun 2008 & Jun 2009 — **Collaborative project**
Prof. Yves LeConte, Abeilles et Environment, INRA PACA Avignon (France)

Feb 2005 – Jun 2005 — **Semester abroad**
Prof. Alison Mercer, Department of Zoology, University of Otago (New Zealand)

Teaching Experiences

since Aug 2006	Co-supervision of Bachelor, Master, Diploma theses and student internships, Zoology II, University of Würzburg (Germany)
since Aug 2006	Supervision of undergraduate student courses in neurobiology, animal physiology and behavioral physiology Zoology II, University of Würzburg (Germany)
Apr 2006 – Jul 2006	Teaching student assistant in practical courses for bachelor students in vegetation ecology and plant taxonomy, University of Würzburg (Germany)

Awards & Grants

Aug 2011	Invitation to the „International short course on RNAi mediates functional genetics in honey bees", Pennsylvania State University, USA (Grant funded by "Research Council of Norway")
Feb 2005	DAAD Travel-Grant for Semester abroad, University of Otago, NZ

Scientific initiatives

May 2008 – Jul 2009	Organizational committee of the 20th Neuro-DoWo 2009 (neurobiology PhD student workshop) in Würzburg

Miscellaneous

Jan 2012 – Sep 2012	Parental leave (fulltime)

Publication list

Full Paper (Peer-Reviewed)

Muenz TS, Groh C, Maisonnasse A, Le Conte Y, Plettner E, Rössler W. 2015. Neuronal plasticity in the mushroom body calyx during adult maturation in the honeybee and possible pheromonal influences. **under revision**

Muenz TS, Maisonnasse A, Plettner E, Le Conte Y, Rössler W. 2012. Sensory reception of the primer pheromone ethyl oleate. **Naturwissenschaften** 99:421-425

Pasch E*, **Muenz TS***, Rössler W. 2011. CaMKII is differentially localized in synaptic regions of Kenyon cells within the mushroom bodies of the honeybee brain. Journal of Comparative Neurology, **Journal of Comparative Neurology** 519:3700-3712
* equally contributing first authors

Hourcade B, **Muenz TS**, Sandoz JC, Rössler W, Devaud JM. 2010. Long-term memory leads to synaptic reorganization in the mushroom bodies: a memory trace in the insect brain?. **Journal of Neuroscience**, 30: 6461-6465

Stieb SM, **Muenz TS**, Rössler W. 2010. Visual experience and age affect synaptic organization in the mushroom bodies of the desert ant *Cataglyphis fortis*. **Developmental Neurobiology**, 70: 408-423

Conference Abstracts (Poster)

Muenz T, Groh C, Rössler W. 2007. Plasticity of synaptic microcircuits in the mushroom-body calyx of the honeybee brain. Poster Abstracts of the Seventh Göttingen Meeting of the German Neuroscience Society 2007; Göttingen, Germany

Muenz T, Groh C, Rössler W. 2007. Structural plasticity of synaptic complexes in the mushroom- bodies of the honeybee. Poster Abstracts of the Eighth International Congress of Neuroethology 2007; Vancouver, Canada

Muenz T, Rössler W. 2007. Experience dependent plasticity of synaptic complexes in the mushroom bodies of the honeybee brain. Poster Abstracts of the Kleinsthirn-Konferenz 2007 - "Insect Brain and Control of Behaviour"; Tutzing, Germany

Muenz TS, Rössler W. 2008. Plasticity of synaptic complexes in the mushroom-bodies of the honeybee brain depends on age, experience and season. Abstracts of the 6th FENS Forum of European Neurosciences; Geneva, Switzerland

Muenz TS and Rössler W. 2009. Plasticity Smells like nurse bee spirit?. Poster Abstracts of the Eighth Göttingen Meeting of the German Neuroscience Society 2009; Göttingen, Germany

Muenz TS and Rössler W. 2010. Pheromonal regulation of behavioural. Abstracts of the XVI IUSSI Conference; Kopenhagen, Denmark

Muenz TS, Maisonnasse A, Plettner E, Le Conte Y, Rössler W. 2011. Sensory reception and transmission of the primer pheromone ethyloleate in the honeybee. Poster Abstracts of the Ninth Göttingen Meeting of the German Neuroscience Society 2011; Göttingen, Germany

Muenz TS, Groh C, Maisonnasse A, Grübel K, Le Conte Y, Rössler W. 2013 Pheromonal regulation of synaptic plasticity in the mushroom-body calyx during adult behavioral maturation in the honeybee. Poster Abstracts of the Ninth Göttingen Meeting of the German Neuroscience Society 2013; Göttingen, Germany

Conference Abstracts (Talk)

Muenz T. 2008. "Smells like nurse bee spirit" - Pheromonal modulation of neuronal and behavioural plasticity in the honeybee *(Apis mellifera)"*. Abstracts of the 19th Neuro-DoWo - Neurobiology PhD student workshop 2008; Saarbrücken, Germany

Muenz T. 2008. Pheromonal modulation of neuronal and behavioural plasticity in the honeybee (*Apis mellifera*). Seminar: Entwicklung und Plastizität des Insektennervensystems; Rauischholzhausen, Germany

Muenz T. 2008. Pheromonal modulation of neuronal and behavioural plasticity in the honeybee (*Apis mellifera*). Annual Meeting of the SFB 554; Fabrikschleichach, Germany

Muenz TS. 2009. "Pheromonal regulation of behavioural plasticity in the honeybee". Seminar: Entwicklung und Plastizität des Insektennervensystems; Rauischholzhausen, Germany

Muenz TS and Rössler W. 2009. Pheromonal regulation of behavioural plasticity in the honeybee. 11th European Symposium on Insect Taste and Olfaction; Villasimius, Italy

Muenz TS. 2011. A potential role of synapsin in the synaptic plasticity of the mushroom body calyx. „International short course on RNAi mediates functional genetics in honey bees", Pennsylvania State University, USA

Acknowledgments

Der Weg von der letzten Danksagung bis zu dieser war länger als ich mir das ursprünglich vorgestellt hatte, aber ich hatte eine tolle Zeit und möchte den verschiedenen Personen die dazu beitragen haben meinen Dank aussprechen:

Der größte Dank gebührt sicherlich meinem Doktorvater und Mentor **Wolfgang**, der nie den Glauben an mich verloren hat und mir immer deutlich meine Stärken vor Augen geführt hat. Er hat mich in jeder Hinsicht, sei es durch die Möglichkeit auf Konferenzen fahren zu können, die Herstellung eines Kontakts für eine Kooperation oder einfach nur durch das „Ja“ zu einer Projektidee dabei unterstützt meinen Weg im Grenzbereich der Neuro-etho/öko-logie zu finden und meine Selbstständigkeit zu entwickeln.

Auch meiner „Doktortante“ **Alison** möchte ich danken. Sie hat nicht nur als Erste mein Interesse für die Neuroanatomie der Honigbienen geweckt, sondern hat mir auch stets das Gefühl gegeben, dass Neuseeland nicht am anderen Ende der Welt ist wenn ich mal ein Anliegen hatte.

Wenn man etwas länger dabei ist, gibt es umso mehr Menschen denen man seine Wertschätzung ausdrücken möchte:

Mit **Claudia** verbindet mich nun schon eine so lange räumlich wie geistige Nähe, dass wir uns oft blind verstehen - ich kann mir keine angenehmere Zusammenarbeit vorstellen. Wichtige Wegbegleiter der letzten Jahre war meine Ur-Doktoranden Generation. **Andi, Kinne, Martin, Sara und Timo** haben nicht nur mit vielen tollen Gesprächen, „Kaffee, Kippen, Cola“, Frisbee und Papierkorb-Basketball unvergessliche Erinnerungen in einer einzigartigen Zeit geprägt, sondern sind echte Freunde geworden. Auch von den jetzigen Mitgliedern der Neuroethologie-Gruppe - **Jan, Tina, Franzi, Anne, Maren, Pauline, Frank, Leonie, Nils, Johannes, Martin, Ayse, Agustina** - möchte ich absolut niemanden missen. Durch ihre witzige, spontane, unkomplizierte aber auch individuelle Art, egal ob im Labor, am Berg, beim Vortrag, beim Grillen oder auf einer Konferenz machen sie die Zoologie II zu einem der erstrebenswertesten Arbeitsplätze die man sich nur vorstellen kann. Dazu tragen natürlich **auch alle weiteren Mitarbeiter der Zoologie II** bei, die den Arbeitsalltag durch spannende, geistreiche und lustige Episoden bereichern und abwechslungsreich gestalten.

Bedanken möchte ich mich auch bei **Konny, Dirk** und **Malu** ohne deren technische Expertise viele meiner Ideen, niemals das Stadium eines tatsächlichen Projektes erreicht hätten - Danke für all die Bienen und Mikroglomeruli!

Ich durfte viele Studenten betreuen, aber dreien - **Tina, Jan und Elli** - die früh an meinen Projekten beteiligt waren und ohne deren Einsatz diese Arbeit sicherlich immer noch nicht existent wäre, gebührt ein ganz besonderer Dank.

Ein großes Dankeschön auch an **all meine Freunde**, die das Leben „beyond science“ so lebenswert machen.

Der Dank den ich **meiner Familie** schulde ist wieder einmal nur schwer in Wort zu fassen: Meine **Eltern**, haben mich während meines ganzen Lebens bedingungslos bei der Umsetzung meiner Träume unterstützt, so auch bei meiner Doktorarbeit. Vor allem aber meiner lieben Frau **Sandra** und meinen tollen Kindern **Jannes** und **Juna** mit ihrer unendlichen Geduld und ihrem unerschütterlichen Glauben an mich, verdanke ich, dass ich nun tatsächlich die letzte Seite meiner Dissertation verfasse.

Wie ich es schon in meiner Diplomarbeit getan habe möchte ich auch meine Doktorarbeit mit einem Comic schließen, der viel Wahrheit über das Verfassen einer Abschlussarbeit kundtut: