Cellular and molecular mechanisms contributing to behavioral transitions and learning in the honeybee

Zelluläre und molekulare Mechanismen, die zu Verhaltensänderungen und Lernen in der Honigbiene beitragen

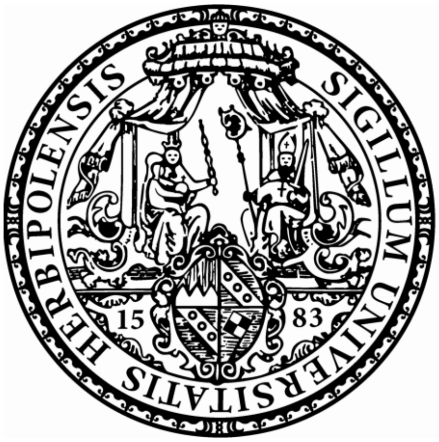

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Table of contents

Summary

The honeybee *Apis mellifera* is a social insect well known for its complex behavior and the ability to learn tasks associated with central place foraging, such as visual navigation or to learn and remember odor-reward associations. Although its brain is smaller than 1mm² with only 8.2 x 10^5 neurons compared to ~ 20 x 10^9 in humans, bees still show amazing social, cognitive and learning skills. They express an age – related division of labor with nurse bees staying inside the hive and performing tasks like caring for the brood or cleaning, and foragers who collect food and water outside the hive. This challenges foragers with new responsibilities like sophisticated navigation skills to find and remember food sources, drastic changes in the sensory environment and to communicate new information to other bees. Associated with this plasticity of the behavior, the brain and especially the mushroom bodies (MBs) - sensory integration and association centers involved in learning and memory formation – undergo massive structural and functional neuronal alterations. Related to this background my thesis on one hand focuses on neuronal plasticity and underlying molecular mechanisms in the MBs that accompany the nurse – forager transition.

In the first part I investigated an endogenous and an internal factor that may contribute to the nurse - forager phenotype plasticity and the correlating changes in neuronal network in the MBs: sensory exposure (light) and juvenile hormone (JH). Young bees were precociously exposed to light and subsequently synaptic complexes (microglomeruli, MG) in the MBs or respectively hemolymph juvenile hormone (JH) levels were quantified. The results show that light input indeed triggered a significant decrease in MG density, and mass spectrometry JH detection revealed an increase in JH titer. Interestingly light stimulation in young bees (presumably nurse bees) triggered changes in MG density and JH levels comparable to natural foragers. This indicates that both sensory stimuli as well as the endocrine system may play a part in preparing bees for the behavioral transition to foraging.

Considering a connection between the JH levels and synaptic remodeling I used gene knockdown to disturb JH pathways and artificially increase the JH level. Even though the knockdown was successful, the results show that MG densities remained unchanged, showing no direct effect of JH on synaptic restructuring.

To find a potential mediator of structural synaptic plasticity I focused on the calcium-calmodulin-dependent protein kinase II (CaMKII) in the second part of my thesis. CaMKII is a protein known to be involved in neuronal and behavioral plasticity and also plays an important part in structural plasticity reorganizing synapses. Therefore it is an interesting candidate for molecular mechanisms underlying MG reorganization in the MBs in the honeybee. Corresponding to the high abundance of CaMKII in the learning center in vertebrates (hippocampus), CaMKII was shown to be enriched in the MBs of the honeybee. Here I first investigated the function of CaMKII in learning and memory formation as from vertebrate work CaMKII is known to be associated with the strengthening of synaptic connections

inducing long term potentiation and memory formation. The experimental approach included manipulating CaMKII function using 2 different inhibitors and a specific siRNA to create a CaMKII knockdown phenotype. Afterwards bees were subjected to classical olfactory conditioning which is known to induce stable long-term memory. All bees showed normal learning curves and an intact memory acquisition, short-term and mid-term memory (1 hour retention). However, in all cases long-term memory formation was significantly disrupted (24 and 72 hour retention). These results suggests the necessity of functional CaMKII in the MBs for the induction of both early and late phases of long-term memory in honeybees. The neuronal and molecular bases underlying long-term memory and the resulting plasticity in behavior is key to understanding higher brain function and phenotype plasticity. In this context CaMKII may be an important mediator inducing structural synaptic and neuronal changes in the MB synaptic network.

Zusammenfassung

Die Honigbiene *Apis mellifera* ist ein soziales Insekt, das bekannt ist für sein komplexes Verhalten und seine Fähigkeiten, Aufgaben in Bezug auf zentrales Sammelverhalten, zum Beispiel visuelle Navigation oder die Assoziation Duft – Belohnung, zu lernen, ist. Obwohl das Bienengehirn kleiner als 1mm² ist und im Vergleich zum dem des Menschen mit ~ 20 x 109 Neuronen nur 8.2 x 105 Neurone besitzt, verfügen Bienen trotzdem über beeindruckende soziale, kognitive und Lernfähigkeiten. Sie praktizieren eine altersabhängige Arbeitsteilung mit Ammen, die im Stock bleiben und Aufgaben wie das Versorgen der Brut übernehmen, und Sammlerinnen, die außerhalb des Stockes Futter und Wasser suchen. Dies fordert die Sammlerinnen zu neuen Aufgaben heraus, zum Beispiel hochentwickelte Navigation, drastischen Änderungen der sensorischen Umwelt, Lernen neuer Assoziationen und die Vermittlung der neuen Informationen an andere Bienen. Diese phänotypische Plastizität geht mit stark strukturellen und funktionell neuronalen Veränderungen im Gehirn und vor allem in den Pilzkörpern – sensorische Integrierungszentren, die an Lernen und Gedächtnisbildung beteiligt sind – einher. Passend dazu liegt ein Schwerpunkt meiner Arbeit darauf, die neuronale Plastizität und die molekularen Mechanismen im Pilzkörper, die mit der Wandlung der Amme hin zur Sammlerin zusammen hängen, zu untersuchen.

Im ersten Teil werden ein endogener und ein interner Faktor, die zum Ammen - Sammlerinnen Übergang und den damit einhergehenden Änderungen im neuronalen Netzwerk beitragen könnten, untersucht: sensorische Input (Licht) und Juvenilhormon (JH). Junge Bienen wurden frühzeitig dem Licht ausgesetzt und anschließend synaptische Komplexe (Mikroglomeruli, MG) in den Pilzkörpern beziehungsweise JH aus der Hämolymphe quantifiziert. Die Ergebnisse zeigen, dass der Einfluss des Lichts tatsächlich eine plastische Verringerung der MG-Dichte auslöst und massenspektrometrische Messungen eine Zunahme an der JH-Menge in der Hämolymphe zeigen. Interessanterweise führt die Stimulation der jungen Ammen mit Licht zu Änderungen in der MG-Dichte und zu JH-Mengen, die vergleichbar sind mit den Werten bei natürlichen Sammlerinnen sind. Dies weist darauf hin, dass sowohl sensorische Stimuli als auch das Hormonsystem einen Beitrag zu der Vorbereitung der Bienen auf die bevorstehende Verhaltensänderung leisten.

Um eine Verbindung zwischen der JH-Menge und synaptischen Umstrukturierungen in Betracht zu ziehen, habe ich einen Gen-Knockdown eingesetzt, um JH-Signalwege zu manipulieren und dadurch die JH-Menge künstlich zu erhöhen. Obwohl der Knockdown erfolgreich war, zeigen die Ergebnisse keinen direkten Zusammenhang zwischen der JH-Menge und einer synaptischer Umgestaltung.

Um einen möglichen Vermittler von struktureller Plastizität zu finden, habe ich den Fokus im zweiten Teil meiner Arbeit auf die Calcium-Calmodulin-abhängige Protein-Kinase II (CaMKII) gerichtet. CaMKII ist ein Protein, das für seine Rolle sowohl in neuronaler und Verhaltensplastizität als auch in struktureller Plastizität bekannt ist. Daher ist es ein interessanter Kandidat, um molekulare

Mechanismen zu untersuchen, die bei der MG-Umstrukturierung in den Pilzkörpern beteiligt sind. In Übereinstimmung mit dem hohen Vorkommen der CaMKII in Lernzentren in Vertebraten (Hippocampus) kommt CaMKII auch in hohem Maß in den Pilzkörpern der Biene vor. In dieser Arbeit habe ich zuerst die Funktion der CaMKII in Lernvorgängen und bei der Gedächtnisbildung untersucht, da bekannt ist, dass CaMKIII mit verstärkten synaptischen Verbindungen, die Langzeitpotenzierung und Gedächtnisbildung auslösen, in Zusammenhang gebracht wird. Der experimentelle Ansatz beinhaltet die Manipulation der CaMKII mit zwei verschiedenen Inhibitoren und einer spezifische siRNA, um einen CaMKII-Knockdown-Phänotyp zu erzeugen. Alle Substanzen wurden über den medialen Ocellartrakt injiziert, um zu gewährleisten, dass sie den Pilzkörper erreichen. Anschließend wurde eine klassische olfaktorische Konditionierung durchgeführt, die ein stabiles Langzeitgedächtnis induziert. Alle Bienen zeigten ein normales Lernverhalten, Kurzzeitgedächtnis und Mittelzeitgedächtnis (eine Stunde Speicherung) waren intakt. Jedoch war in allen Fällen das Langzeitgedächtnis beschädigt (24 und 72 Stunden Speicherung). Diese Ergebnisse legen nahe, dass CaMKII in den Pilzkörpern essentiell für das Auslösen von frühen und späten Formen des Langzeitgedächtnisses der Biene ist. Die neuronalen und molekularen Grundlagen des Langzeitgedächtnis sind der Schlüssel, um höhere Gehirnfunktionen und phänotypische Plastizität zu verstehen. CaMKII könnte ein wichtiger Vermittler sein, um strukturelle und neuronale synaptische Änderungen im Netzwerk des Pilzkörpers auszulösen.

General introduction

The social lifestyle of the honeybee

The highly social lifestyle enables insects like bees, ants and wasps to be successful in colonizing almost all terrestrial habitats in the world (Hölldobler and Wilson 1990; Schultz 2000). Honeybee colonies may comprise up to 40.000 individuals and their ability to adapt to a variety of habitats are particularly attributed to their social lifestyle enabling them to collaborate to sustain the colony (Lindauer 1952). Division of labor, overlapping generations and the cooperative care of the young are the three key criteria Edward O. Wilson described 1971 as the basis for eusociality. A honeybee colony is divided in reproductive castes with one female queen and several hundred male drones (during mating season) and a worker caste with 10,000 – 40,000 females (Winston 1987). The workers rely on the queen controlling the colony demography and producing offspring, whereas the queen relies on the workers collecting food, taking care of the brood, building and maintaining the combs, guarding the hive and various other duties (Wilson, 1971). Within the female worker caste honeybees show an age-related division of labor (reviewed in Robinson 1992). For the first three weeks honeybee workers perform tasks inside the hive. They are called nurse bees, as one of their most important tasks is caring for the brood. Nests of the European honeybee usually are constructed in dark cavities. Therefore olfactory and tactile information and communication dominate within the hive for the nurses. When the bees leave the hive for the first time, they perform orientation flights to learn the location of the hive and the local landscape (Lindauer 1952; Lindauer 1961, von Frisch 1967; Vollbehr 1975; Zeil et al. 1996). During the orientation flights bees circle in loops to orient in the closer surroundings from different viewpoints which is crucial for their navigation skills (Becker 1958; Capaldi and Dyer 1999; Capaldi et al. 2000; Degen et al. 2015). After this initial phase to learn the location of the hive and the surroundings they start to go on longer flights and collect food and water to maintain the colony as forager bees. With this switch from indoor to outdoor duties the bees' focus shifts from mainly olfactory communication to the suddenly more prominent visual input. They have to rely on visual stimuli like manifold colored blossoms and flowers, the daily course of the sun, polarized skylight patterns and landmarks to use this information in orientation and navigation to forage successfully .g. (e.g. Wilson 1971; Michener 1974; Menzel et al. 1996; Collett and Collett 2002; Menzel et al. 2010; Srinivasan 2006). Under normal conditions, bees perform nurse duties for about 3 weeks and then forage for approximately 2 – 3 weeks (Wilson 1971). However, age-related division of labor is not rigid but rather flexible to respond to the actual environmental conditions and the corresponding needs of the hive (Rösch 1925; Robinson 1992; Huang and Robinson 1996). For example, by changing the age demography of a colony creating a deficiency of nurses, foragers reverse to nurse duties. Vice versa depriving a colony of foragers leads to a precocious start of foraging in nurse bees (Rösch 1930;

Robinson 1992). These flexible behavioral adaptations allows the age–related division of labor to be a highly successful system that is always able to react to environmental changes.

The mushroom bodies – centers for sensory integration, learning and plasticity

Structure of the honeybee brain and the mushroom bodies

The central honeybee brain is subdivided into three main divisions: protocerebrum, deutocerebrum and tritocerebrum. The deutocerebrum includes the antennal and the dorsal lobes, the tritocerebrum consists of two bilateral lobes and the protocerebrum comprises the optic lobes, the central complex, the lateral horn, the protocerebral lobes and the mushroom bodies (MB) (Ito et al. 2014). The most prominent neuropils within the dorsal protocerebrum are the MBs, two bilaterally symmetric structures in both hemispheres of the brain (Mobbs 1982; Strausfeld et al. 1998; Strausfeld 2002; Fahrbach 2006). The MBs are formed by ~368.000 intrinsic neurons (Witthöft 1967; Strausfeld 2002; Fahrbach 2006) the so called Kenyon cells (KCs) (Kenyon 1986), which represent an astonishing ~43% of the 960,000 neurons of the whole honeybee brain (Menzel and Giurfa 2001; Rössler and Groh 2012). The KCs form the structural architecture of the MB. The cup shaped calyces are built by KC dendritic arborizations, the peduncle is shaped by axonal projections, and KC cell bodies surround and fill the calyx cups (see fig. 1) (Mobbs 1982). In Hymenoptera at least three populations of KCs can be differentiated by their distribution and size: the inner compact cells (IC) with a small diameter in the center of the calyx cup, the inner non-compact cells (NC) with a large diameter lying at the inner sides of the calyx cup, and the outer compact cells (OC) situated around the calyx cup (see fig. 1B) (Mobbs 1982; Farris et al. 1999). The calyces are the main input zone of the MBs and receive mainly cholinergic signals from projection neurons (PN) of primary sensory neuropils (Strausfeld et al. 1998). Additionally they are innervated by modulatory input from GABAergic feedback neurons (Bicker et al. 1985; Grünewald 1999; Ganeshina and Menzel 2001; Froese et al. 2014).

The different KC subpopulations innervate distinct areas in the MB calyx that are anatomically and functionally partitioned: the lip, the collar and the basal ring (see fig. 1B) (Mobbs 1982; Strausfeld 2002). Lip and collar are innervated by the dendritic network of the NCs (Mobbs 1982), the basal ring by the ICs and the dendrites of the OCs branch in all three subcompartments (Mobbs 1982; Strausfeld 2002). Sensory afferents from PNs functionally innervate the three regions. The lip receives olfactory input from the antennal lobes (Mobbs 1982; Sun et al. 1997; Abel et al. 2001; Gronenberg 2001; Kirschner et al. 2006), the collar visual input from lobula and medulla (Mobbs 1982; Gronenberg 2001), and both sensory modalities are represented in the basal ring (Ehmer and Gronenberg 2002). Besides vision and olfaction, proboscis sensory afferents (most likely gustatory and mechanosensory) are represented in parts of the upper collar and the basal ring (Schröter and Menzel 2003). As honeybees show a dominance of visual information processing in the brain, the MB collar represents the largest

compartment in the calyx compared to for example ants that heavily rely on olfactory cues and correspondingly possess a enlarged lip region (Durst et al. 1994; Withers et al. 1995; Ehmer et al. 2001). In the MB calyx synaptic connections between the PNs and the KCs form distinct synaptic complexes, the so called microglomeruli (MG) (Gronenberg 2001; Frambach et al. 2004). Each MG contains a large presynaptic PN bouton, surrounded by KC dendritic spines (see fig. 1, insert in A) (Ganeshina and Menzel 2001; Frambach et al. 2004; Ganeshina et al. 2006; Groh et al. 2004, 2006; Scholl et al. 2014). The output region of the calyces is formed by KC axonal projections that run parallel in one peduncle in each hemisphere and arborizes in the vertical and the medial lobes (Ito et al. 2014). The fibers of the vertical lobe are organized in three layers corresponding to the calyx subcompartments lip, collar and basal ring. An additional fourth layer - the γ lobe - contains axons of OCs (Strausfeld 2002).

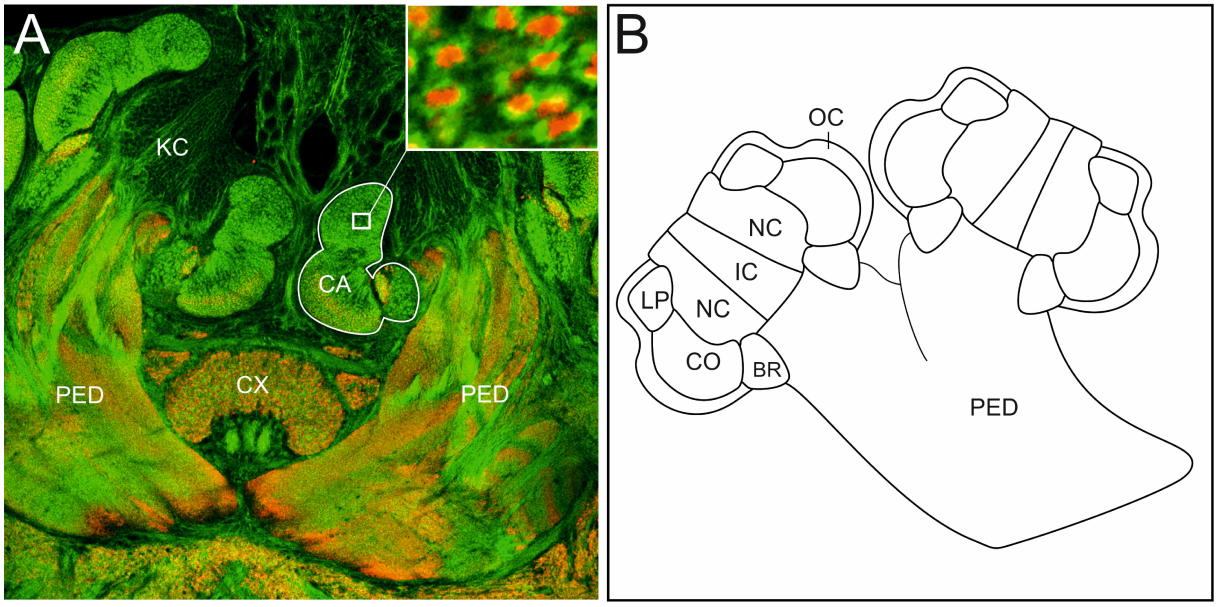

Figure 1: Structure of the mushroom bodies (MBs)

A: Double-labeling for synapsin (red) and f-actin (green) visualizing the central brain neuropils including central body (CX), peduncles (PED), Kenyon cells (KC) and calyces (CA). Insert: Synaptic complexes (microglomeruli = MG) consisting of presynapse (red) and postsynaptic partners (green) enlarged from the lip region **B**: Schematic drawing of a MB including the peduncles (PED) and the calyx with its subcompartements basalring (BR), collar (CO) and lip (LP). The inner compact KCs (IC) are located in the center of the calyx cups, the inner non compact KCs (NC) at the sides of the cup, and the outer compact KCs outside the calyces.

Learning and plasticity in the mushroom bodies

Honeybees possess remarkable learning and memory capabilities (reviewed, for example, in Menzel et al. 2006). For being successful foragers need the ability to learn and memorize for example the locations of profitable food sources and the position of the hive (von Frisch 1967; Collett and Collett 2002; Zhang et al. 1996; Menzel 2009). Under laboratory conditions this amazing ability to learn and associate different stimuli can be used to study the molecular bases of learning and memory formation.

One learning assay that is commonly used and well established in honeybees is an associative olfactory conditioning protocol using the proboscis extension response (PER) (Takeda 1961; Bittermann et al. 1983; reviewed in Giurfa and Sandoz 2012). In this protocol the PER is paired with a sugar reward (unconditioned stimulus = US) whilst the bees are simultaneously exposed to an odor (conditioned stimulus = CS). The bees learn to associate the US with the CS and afterwards show PER in response to the odor alone (Takeda 1961; Bittermann et al. 1983). The association of three or more pairings is sufficient to induce long-term memory (LTM) that can last for the honeybees' lifetime (Menzel 1999; Hourcade et al. 2009). In honeybees the memory phases can be separated into short-term memory, lasting only seconds, mid-term memory (MTM), lasting for several hours, early LTM (eLTM), lasting 1 - 3 days and late LTM (lLTM), that can be retrieved three or more days after conditioning (Menzel 1999). The neuropil in the honeybee brain that is known for its importance in learning and memory processes is the MB (reviewed, for example, in Menzel and Giurfa 2001; Heisenberg 2003; Fahrbach 2006). Hourcade et al. 2010 showed that following olfactory learning using PER and formation of a stable olfactory long term memory, changes in the density of MG occurred in the MB calyx. Similar results were found in leaf-cutter ants after aversive conditioning and the formation of an aversive long term memory (Falibene et al. 2015).

The MBs including the MG have not only been shown to be plastic during memory formation, they are also able to adapt to changes in the lifestyle and the behavior of the bees (Groh and Rössler 2008; Rössler and Groh 2012). One important point in the honeybees' life - the transition from nursing to foraging - is accompanied by various changes in their sensory environment that are reflected in alterations in the brain. In foragers the overall brain volume is increased compared to nurses (Durst et al. 1994; Fahrbach and Moore 1998; Ismail et al. 2006). This increase is most prominent within the MBs which show 14.8% higher volume in foragers compared to one day old bees (Erber et al. 1980; Withers et al. 1993; Durst et al. 1994; Strausfeld et al. 1998). Especially the visually innervated collar shows a prominent volume increase during the transition to outdoor foragers (Durst et al. 1994), indicating the sudden importance of visual inputs associated with tasks outside the hive. On the synaptic level it could been shown that the observed volume changes are accompanied by distinct changes in MG density in the MB calyces possibly due to dendritic growth of KC dendrites and pruning of PN boutons (Farris et al. 2001; Groh et al. 2006, 2012; Scholl et al. 2014; Muenz et al. 2015). Importantly the observed changes in different age groups are not strictly age-related, but include experience-independent and experience-dependent components (Fahrbach and Moore 1998). The changes observed with increasing age rather depend on the foraging experience of the individual bees (Withers et al. 1993; Groh et al. 2012; Scholl et al. 2014). In the first week of a bee's adult life the increase in MB volume is most distinct (Muenz et al. 2015) which could indicate an internal age-related program (Farris et al. 2001; Ismail et al. 2006; Muenz et al. 2015) whereas later on an external experience-related program

could allow flexible adaptation to factors like experience (Fahrbach and Moore 1998), learning (Hourcade et al. 2010) and sensory stimuli (Scholl et al. 2014). Foraging experience correlates with increased KC dendritic branching contributing to the observed volume increase (Farris et al. 2001; Dobrin et al. 2011). Studies in other social insects confirm these observations, as they also show an age – and experience related MB volume increase (*Camponotus floridanus*: Gronenberg et al. 1996; *Cataglyphis bicolour:* Kühn-Bühlmann and Wehner 2006; *Polybia aequatorialis*: O'Donnell et al. 2004; *Cataglyphis fortis*: Stieb et al. 2010).

Juvenile hormone – a mediator for phenotype plasticity

In insects three hormones are suggested to act as behavioral modulators which contribute to division of labor in honeybees: ecdysteroids, insulin and juvenile hormone (JH) (reviewed in Bloch 2009).
In adult insects ecdysteroid takes part in differentiating the castes by playing a role in regulating oogenesis, vitellogenesis and sex pheromone production (Hagedorn 1985). Insulin / insulin-like growth factor is associated with a variety of functions contributing to division of labor like the transition from nurse to forager (Ament et al. 2008), caste determination between queen and worker (Patel et al. 2007; Wheeler et al. 2006), nectar preference during foraging (Hunt et al. 2007) and longevity (Corona et al. 2007). The last hormone - JH – is maybe the most studied of the three and plays an important role during insect development and especially metamorphosis (reviewed in Robinson and Vargo 1997). In many insects high JH titers prevent the induction of metamorphosis, whereas high titers are needed prior to each ecdysis and low titers are essential for the transformation of the pupa to the imago (reviewed in Riddiford 1996). In contrast, in adult honeybees JH does not seem to act as a classical gonadotropin as it is not involved in adult oogenesis and displays low titers in reproductive active queens (reviewed in Bloch 2009). It rather plays a role in controlling behavior by regulating reproductive division of labor between queen and workers as well as age-related division of labor in sterile workers (Robinson 1992; Robinson and Vargo 1997; Bloch et al. 2002; reviewed in Bloch et al. 2009).

Regulation of JH

JH is synthesized in the paired corpora allata and released into the hemolymph where it can be directly regulated by factors circulating in the hemolymph and indirectly by innervation via neurosecretory cells which are located in the brain (reviewed in Tobe and Stay 1985).
In many insects the corpora allata itself and thereby JH production is regulated by neuropeptides including allatostatins and allatotropins (reviewed for example by Stay and Tobe 200; Hiruma and Kaneko 2013). Allatostatins are thought to inhibit JH synthesis and secretion (Stay and Tobe 2007), whereas allatotropin may increase JH level (for example Elekonich and Horodyski 2003). However the

role of allatotropin remains controversial, as for example Kaneko and Hiruma (2015) showed a downregulation of JH due to an indirect effect of allatotropin in *bombyx mori*. So far the regulation of the corpora allata and the role of neuropeptides in regulating it remains unknown in the honeybee and are needed to be analyzed in the future to better understand the pathways including JH.

One important JH regulator circulating in the hemolymph is the yolk precursor protein vitellogenin (Vg). It is synthesized in the abdominal fat body cells (Engels 1974; Chapman 1998) and secreted into the hemolymph where it is a predominant protein (Fluri et al. 1982). Vg and JH show an inverse pattern and mutually suppress each other (Hartfelder and Engels 1998; Pinto et al. 2000; Amdam and Omholt 2003; Guidugli et al. 2005). An RNAi mediated *vg* knockdown was shown to increase JH synthesis (Amdam et al. 2003; Guidugli et al. 2005 Wang et al. 2012) and vice versa high levels of JH inhibit Vg protein expression (Fluri et al. 1982; Fahrbach and Robinson 1996; Pinto et al. 2000). Additionally, treatment with the JH analog methoprene leads to reduced Vg expression in workers and queens (Corona et al. 2007).

Another substance group that can influence JH levels in honeybees are pheromones. Brood pheromone and honeybee queen mandibular pheromone can delay foraging activity in workers (Le Conte et al. 2001; Pankiw et al. 1998a). Interestingly both are thought to influence division of labor via regulation of JH level (Pankiw et al. 1998b; Kaatz et al. 1992; Le Conte et al. 2001).

Role of JH during the nurse - forager transition

Division of labor in female worker honeybees is distinguished by the transition from indoor working nurses to forager bees who collect food and water outside the hive. This behavioral transition is accompanied by a rise in juvenile hormone (JH) (Robinson et al. 1989; Robinson 1992; Huang et al. 1991, 1994), with young nurses showing low levels and foragers expressing the highest level of JH within the colony (Huang et al. 1991, 1994; Scholl et al. 2014; Robinson et al. 1989). Age-related division of labor in honeybees is a very flexible process and factors like environmental changes, nutritional status of the colony and colony age demography all take part in a flexible nurse – forager workforce distribution (Robinson 1992; Huang and Robinson 1992, 1996). Correspondingly foragers that "reverse" to nurse duties show a drop in JH titers (Robinson et al. 1989; Robinson 1992; Huang and Robinson 1992, 1996) and treatment with JH analog induces precocious foraging (Jaycox et al. 1974, Jaycox 1976; Robinson 1985, 1987). Notably, JH is upregulated prior to the transition to foraging and is not a result of foraging experience (Jassim et al. 2000; Elekonich et al. 2001). Taken together with the fact that removal of the corpora allata causes a delayed onset of foraging (Sullivan et al. 2000), these results indicate a role of JH as an important mediator in the timing of the onset of foraging, rather than trigger the onset itself.

As described above Vg regulates JH and vice versa, implying a role for Vg as an important regulator of JH and thereby a mediator for division of labor itself. Genetical approaches using knockdown of *vg*

showed not only a significant increase of JH in the hemolymph (Amdam et al. 2003; Guidugli et al. 2005; Amdam et al. 2007; Wang et al. 2012) but also a precocious onset of foraging of young bees (Guidugli et al. 2005; Amdam et al. 2006; Nelson et al. 2007; Ihle et al. 2010). The 'double repressor hypothesis' proposed by Amdam and Omholt (2003) takes two regulatory processes of Vg into account. JH acts as the first repressor and internally suppresses Vg synthesis. The second repressor works externally and includes the social context of the bee, the season of year and the demographic structure of the hive, factors shown to influence JH level (Robinson et al. 1989; Huang et al. 1991; Huang and Robinson 1995, 1996). Together the two repressors dynamically control the feedback loop to regulate Vg production and eventually also JH levels.

Calcium / calmodulin-dependent kinase II – a mediator for synaptic plasticity

In the search for a molecular mediator of plasticity in neuronal circuits in the brain the calcium / calmodulin – dependent protein kinase II (CaMKII) is a potential candidate. CaMKII contributes up to 2% to the total mass of proteins present in the central nervous system of mammals (Erondu and Kennedy 1985). In vertebrates four closely related genes (CAMK2A, CAMK2B, CAMK2G, and CAMK2D) code for the α, β, γ and δ CaMKII isoforms (Gaertner et al. 2004), with the α and β form occurring predominantly in the brain (Miller and Kennedy 1986). CaMKIIα is the most abundant protein in the post synaptic density in the forebrain (Cheng et al. 2006) and has been extensively studied in association with memory formation (e.g. Elgersma et al. 2002). In the context of its high expression it is not surprising that CaMKII possesses many different and profound roles in long-term potentiation (LTP), synaptic plasticity and memory formation.

CaMKII structure:

CaMKII monomers consist of an N-terminal catalytic domain, an autoinhibitory domain, a self-association domain and a variable segment (see fig. 2a) (Lisman et al. 2002). The catalytic domain contains the ATP binding sites, substrate binding sites and sites for interaction with anchoring proteins. The self-association domain is necessary for the formation of the holoenzymes. 12 CaMKII monomers first assemble to two hexameric rings that together form a holoenzyme (Rosenberg et al. 2005; Hoelz et al. 2003; Kolodziej et al. 2000; Morris and Török 2001). In its initial state the CaMKII is inactive due to the binding of the autoinhibitory domain to the S- and the T-site of the catalytic domain forming a coiled coil structure that blocks the enzymatic activity (see fig. 2b). After neuronal excitation followed by Ca^{2+} influx into the postsynapse Ca^{2+} / Calmodulin (Ca^{2+} / CaM) complexes are formed and bind to CaMKII. Subsequently the inhibitory domain detaches from the catalytic domain exposing threonine 268 (Thr286) that in turn gets phosphorylated by adjacent catalytic domains and thereby activate the enzyme (Morris and Török 2001; Rich and Schulman 1998; Barria et al. 1997). T268 phosphorylation in

turn inhibits the reformation of the coiled coil structure, resulting in autonomous kinase activity (Miller and Kennedy 1986) even if Ca^{2+} / CaM dissociates from the enzyme (see fig. 2b) (Saitoh and Schwartz 1985; Lou et al. 1986; Miller and Kennedy 1986; Yang and Schulman 1999). The duration of this constitutively active state can persist for hours after the Ca^{2+} influx has passed (Fukunaga et al. 1993; Ouyang et al. 1997, 1999). To regulate and deactivate CaMKII endogenous protein phosphatases can dephosphorylate the autoinhibitory sites to switch of the Ca^{2+} independent CaMKII function (Radwańska et al. 2010; Lepicard et al. 2006).

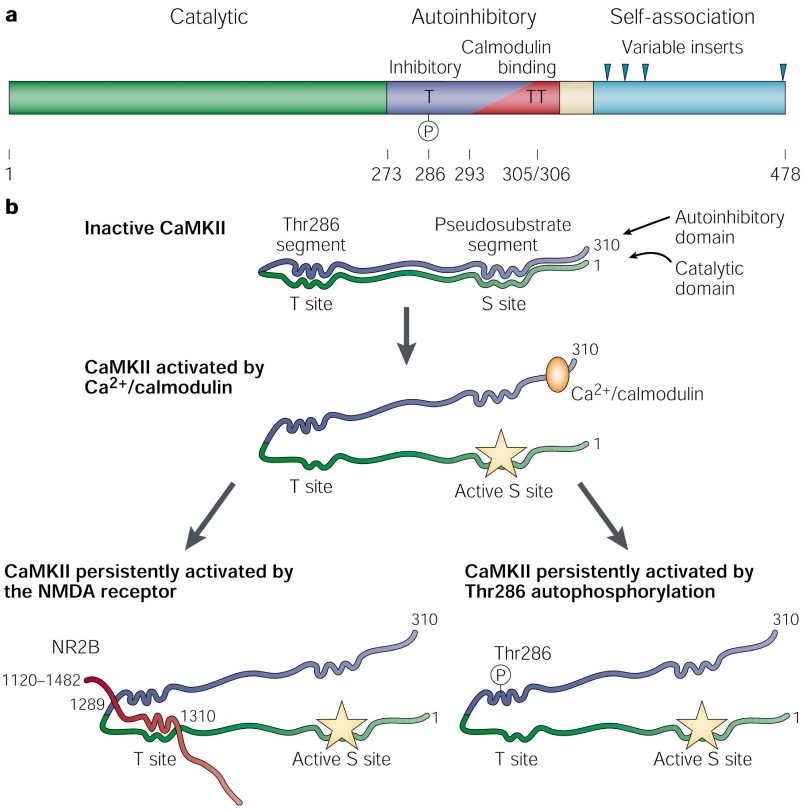

Figure 2: CaMKII structure (adapted after Lisman et al. 2002)

a: The calcium / calmodulin-dependent protein kinase II (CaMKII) contains 3 domains: a catalytic domain, a self-association domain and an autoinhibitory domain that includes different phosphorylation sites **b**: In its inactive state the catalytic and the autoinhibitory domains are joined on the T- and the S- site. After CaMKII activation via binding of calcium / calmodulin the two domains dissociate and the catalytic site can be autophosphorylated on Thr286. Autophosphorylation or binding to the NMDA receptor subunit NR2B both results in a persistent calcium-independent activity of CaMKII.

CaMKII function:

The function of CaMKII has mostly been studied in the CA1 region in the vertebrate hippocampus where CaMKII was shown to be necessary for the induction of LTP (reviewed in Lisman et al. 2012). LTP is defined as a long–term increase in the transmission between 2 neurons. In 1949 even before the term LTP was described Donald Hebb already postulated his well-known theory that simultaneous activity of two neurons will strengthen their connection - "Cells that fire together, wire together". To induce LTP the NMDA receptor (NMDAR) acts as a coincidence detector that only activates after presynaptic release of Glutamate simultaneously with postsynaptic depolarization transmitted via AMPA receptors (AMPAR). The resulting calcium influx through activated NMDAR starts the molecular signaling cascade that induces LTP and thereby enduring strengthening of the synaptic connection between the activated neurons. One important protein taking part in this signal cascade is the CaMKII which is activated due to NMDAR mediated Ca^{2+} influx and was shown to be necessary for mediating LTP (Silva et al. 1992b; Pettit et al. 1994; Wang and Kelly 1995; Otmakhov et al. 1997; Hinds et al. 1998). Genetic and pharmaceutical manipulations targeting the CaMKII regulatory domain in mice resulted in a complete blockade of LTP induction (Malenka et al. 1989; Malinow et al. 1989) and in the same line knock-out mice which lacked the gene for alpha CaMKII could not express LTP (Silva et al. 1992b, 1992a; Elgersma et al. 2002). One mechanism how CaMKII triggers the strengthening of a synapse during LTP is by affecting postsynaptic AMPAR. CaMKII can directly phosphorylate AMPAR at the amino acid GluR1 leading to an increased current flow (Mammen 1997; Walikonis et al. 2001; Barria and Malinow 2005). Additionally CaMKII activation induces the recruitment of new AMPAR to the postsynaptic density (Hayashi et al. 2000), the translocation of AMPAR into spines by exocytose of vesicles (Lledo et al. 1998) and the insertion of AMPAR in the postsynaptic membrane (Srinivasan et al. 1994; Lisman and Zhabotinsky 2001).

CaMKII is not only necessary for the induction of LTP but also for LTP maintenance. But in this case catalytic active CaMKII is not necessarily needed but the unphosphorylated protein may rather function as a synaptic tag guiding other proteins to selectively find potentiated synapses (Frey and Morris 1997; Okamoto et al. 2009). The small fraction of catalytic inactive CaMKII which translocates to the synapse and binds to NMDAR is curcial for LTP maintenance (Barria and Malinow 2005; Feng et al. 2011) and NMDAR / CaMKII complexes are thought to play a role in enlarging synapses during late LTP by acting as a synaptic tag. The complex operates as a binding site for other molecules involved in LTP for example Arc, casein kinase 2 and many more (reviewed in Hell 2014) thereby increasing synaptic structure and strength (Lisman and Raghavachari 2014). Another important tagging function of CaMKII is its part in restructuring the actin dendritic spines cytoskeleton, resulting in increasing spine size after LTP (Rachinsky and Feldlaufer 2000). Inactive αCaMKII can directly bind to f-actin or bundle f-actin through its ß-subunit (Shen and Meyer 1999; Fink et al. 2003; Okamoto et al. 2007; Lin and

Redmond 2008). In its basal inactive state, CaMKII anchors and bundles f-actin (Shen and Meyer 1999; Lin and Redmond 2008). Following Ca^{2+} influx βCaMKII detaches from f-actin (Okamoto et al. 2007; Lin and Redmond 2008) and enables the reorganization of the actin cytoskeleton. After the return of CaMKII to its basal, inactive state CaMKII again binds to actin and stabilizes the changes (see fig. 3) (Okamoto et al. 2007; Lin and Redmond 2008).

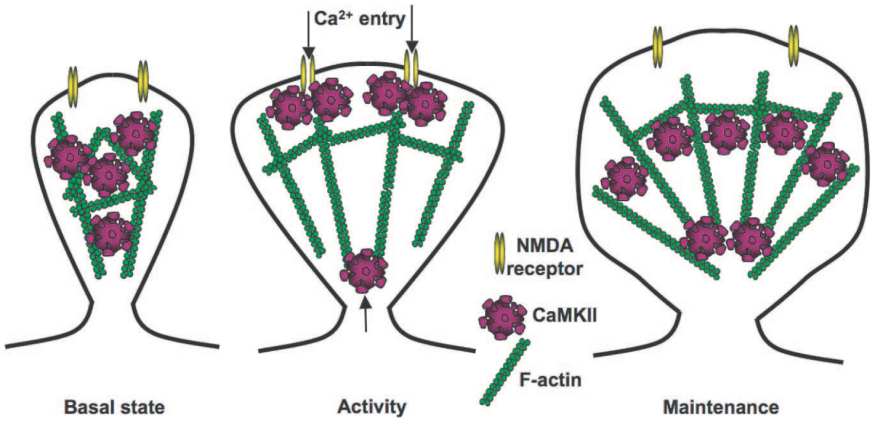

Figure 3: The role of CaMKII in restructuring the synaptic actin skeleton (Okamoto et al. 2007)

In the basal state CaMKII is inactive and binds to f-actin. After synapse excitation and following Ca^{2+} influx CaMKII gets phosphorylated, detaches from f-actin filaments enabling restructuring of the actin cytoskeleton. After CaMKII inactivation it again bundles f-actin to maintain the new formed structure.

CaMKII role in memory formation:

Several studies implicate LTP as the molecular basis for learning and memory formation (reviewed in Martin et al. 2000) and learning processes were shown to explicitly trigger LTP (Gruart et al. 2006; Whitlock et al. 2006). As CaMKII is necessary for LTP induction it could also be involved in learning and memory formation. Indeed, training rats in one-trial inhibitory avoidance tasks increased the enzymatic activity of CaMKII (Izquierdo et al. 1997) and CaMKII knockout in mice results in impaired learning and memory (Silva et al. 1992b; Frankland et al. 2001). Heterozygote knockout mice with a ~ 50% CaMKII expression reduction showed memory impairments 10 – 50 days after training (Frankland et al. 2001) and αCaMKII knockout mice show learning deficits in the Morris water task (Silva et al. 1992a). Pharmaceutical experiments consolidate the results from genetically manipulations. Injection of the CaMKII inhibitor KN-62 into the hippocampus causing full retrograde amnesia of inhibitory avoidance learning in rats (Tan and Liang 1997).

CaMKII in the honeybee and other invertebrates:

In contrast to vertebrates, insects, as far as investigated, possess only one CaMKII gene. However due to alternatively splicing multiple isoforms exist (*Apis melifera*: Altfelder et al. 1991; *Drosophila melangonaster*: Cho et al. 1991; Griffith and Greenspan 1993; Ohsako et al. 1993; *Bomyx mori*: Shanavas et al, 1998) which are accumulated in the MBs (Kamikouchi et al. 2000; Takamatsu et al.

2003; Lent et al. 2007; Pasch et al. 2011). In the honeybee CaMKII mRNA and protein are especially accumulated in the MBs intrinsic neurons (KCs) with an increased enzymatic activity of CaMKII in the KCs compared to other cell types in the honeybee brain (Kamikouchi et al. 2000; Pasch et al. 2011). Evidence for a role of CaMKII in learning and memory formation in insects was first shown in *Drosophila*. Peptide inhibition of CaMKII causes synaptic defects and memory deficits in the courtship - conditioning assay and CaMKII knockout flies show impaired associative learning (Griffith et al. 1993, 1994; Joiner and Griffith 1999). Corresponding to vertebrates an increase of phosphorylated CaMKII after training in a learning paradigm could also be shown in the cockroach (Lent et al. 2007). In honeybees Ca^{2+} is necessary for triggering protein-synthesis dependent LTM (Perisse et al. 2009) and pharmaceutical inhibition of CaMKII disrupted lLTM but not MTM and eLTM (Matsumoto et al. 2014). Studies on crickets using the same inhibitors on the other hand showed a disrupted eLTM but also an intact MTM (Mizunami et al. 2014). Even though there is strong evidence for CaMKII being necessary for learning and memory processes in insects similar to vertebrates, the discrepancies between the studies in honeybees and crickets show that there remain many questions. The mechanism of CaMKII function in memory formation and especially its role in different memory traces need to be investigated in further experiments.

Thesis outline

An individual honeybee cannot mature or survive by itself, but is part of a highly evolved collective engaging in a variety of complex tasks that are not practiced by most solitary insects. Based on an age-related division of labor, each colony member performs a specific job to ensure the survival of the colony as a whole. Thereby the two main behavioral stages a bee passes during its life are on the one hand performing duties inside the hive like taking care of the brood (nurses) and on the other hand gathering food outside the hive (foragers).
Hence the first part of my PhD thesis focuses on studying factors that could contribute to the behavioral nurse – forager transitions trying to answer the following questions:

1) Can sensory input (light) trigger changes in synaptic circuits in the brain?
   Background: Accompanied by the behavioral nurse – forager transition brain volume and synaptic wiring is adjusted in bees. As especially light and vision become more important during foraging light as a sensory stimuli could trigger the observed changes in behavior and in the brain.
   Experimental approach: Measuring the density of synaptic complexes (MG) in the mushroom body calyx after exposing the bees for 3 days to light pulses.
2) Can JH trigger changes in synaptic circuits in the brain and is there a correlation between JH level and light exposure?
   Background: Foragers show an elevated JH level compared to nurse bees and JH is thought to be an important mediator for the onset of foraging.
   Experimental approach: Via knockdown of an antagonist of JH (Vg*)* and a putative JH receptor (*usp*) JH levels were artificially elevated in bees followed by the analysis of the density of MG in the mushroom bodies*.* Additionally JH levels were measured in light exposed and control bees.

The approach and results are discussed in detail in Manuscript I

The second part of my thesis focusses on the amazing learning capabilities of honeybees. With the start of foraging, bees need to be able to orient in a new environment. This includes for example learning the location of the hive and its surroundings, which flowers are rewarding and many other new associations. Therefore the next question focusses on the molecular mechanism of learning and memory formation in the honeybee:

3) Does CaMKII play a role in memory formation in the honeybee?

   Background: CaMKII is a protein known to be necessary for the induction of LTP and was shown to be important for memory formation.

   Experimental approach: Inhibition and knockdown of CaMKII, followed by olfactory conditioning and testing memory retention 1, 24, and 72 hours after learning.

The approach and results are discussed in detail in Manuscript II

Manuscript I: Synaptic reorganization after light exposure

Light Exposure Leads to Reorganization of Microglomeruli in the Mushroom Bodies and Influences Juvenile Hormone Levels in the Honeybee

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Abstract

Honeybees show a remarkable behavioral plasticity at the transition from nursing inside the hive to foraging for nectar and/or pollen outside. This plasticity is a crucial aspect promoting age-related division of labor in honeybee colonies. The behavioral transition is associated with significant volume and synaptic changes in the mushroom bodies (MBs), centers for sensory integration, learning and memory in the honeybee brain. We tested whether precocious exposure to light triggers synaptic remodeling in the MBs. The results show that light pulses over three days induce significant decrease in the density of synaptic complexes (microglomeruli, MG) in visual subregions (collar) of the MB. An increase of juvenile hormone (JH) associated with a decrease in vitellogenin (Vg) appear at the transition from nurse to forager. By disturbing both Vg and JH pathways using gene knockdown of *vg* and ultraspiracle (*usp*), we tested whether these hormones are involved in synaptic remodeling in the MBs. In previous studies, RNAi mediated knockdown of *vg* and *usp* induced an acceleration of the JH level, which can lead to precocious foraging. Our study shows that the MG numbers remained unchanged when Vg and JH pathways were perturbed suggesting no direct influence of hormonal changes on synaptic remodeling. However, mass spectrometry detection of JH revealed that precocious light exposure triggered an increase in JH levels in the hemolymph of young bees. This suggests a dual effect of light exposure via direct effects on synaptic plasticity in the MB calyx and a possible positive feedback on hemolymph JH levels.

Keywords:

microglomeruli; synaptic plasticity; juvenile hormone; division of labor; light exposure

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Introduction

Honeybees (*Apis mellifera*) exhibit a highly complex behavioral repertoire, and honeybee colonies are able to respond in an adaptive manner to changing environmental conditions (eg. Seeley, 1982; von Frisch, 1993).The social organization of honeybee colonies is based on age-related division of labor including a transition from inside duties (nurse bees) to foraging activities outside the hive (foragers) (Lindauer, 1961; Seeley, 1982; Winston, 1987). For the first 2-3 weeks after adult emergence, the bees perform tasks inside the almost dark hive and mostly rely on tactile and olfactory communication and orientation. Then, bees start short orientation flights before they begin to fly out on foraging trips (Winston, 1987; Robinson, 1992). However, division of labor is not a rigid age-dependent process, but rather able to react to changing needs of the colony. For example depriving a colony of nurse bees, results in a reversion of experienced foragers to nurses, to ensure the survival of the brood (Page et al., 1992). With the initiation of orientation flights, visual cues like recognition of landmarks and polarized-skylight patterns become crucial for visually based orientation and navigation (Wilson, 1971; Michener, 1974). The transition from a pheromone-loaded dark hive to the outside world with bright sunlight requires a high degree of sensory and behavioral modification to accommodate the environmental changes. The nurse-forager transition of honeybees, therefore, represents an ideal model to investigate neuronal and hormonal changes underlying behavioral and neuronal plasticity.

The mushroom bodies (MBs) in the insect brain are known as centers for sensory integration and association promoting learning and memory processes (Menzel and Giurfa, 2001; Heisenberg, 2003; Gerber et al., 2004; Giurfa, 2007). In the honeybee the MBs are large, doubled structures containing a substantial proportion of more than 40% of all brain neurons, indicating that in the honeybee the MBs play a substantial role in behavioral plasticity (Chapman, 1998; Strausfeld, 2002; Aso et al., 2009; Rössler & Groh, 2012). Previous studies showed that the transition from nurse bees to foragers is followed by substantial volume increase in the MBs (Withers et al., 1993; Durst et al., 1994; Fahrbach et al., 1998; Farris et al,. 2001). Similar observations were made in Carpenter ants (*Camponotus floridanus;* Gronenberg et al., 1996), desert ants (*Cataglyphis fortis;* Kühn-Bühlmann & Wehner 2006; Stieb et al., 2010, 2012) and some wasps (*Polybia aequatoriali;* O'Donnell et al., 2004) indicating an experience- or task-related increase of the MB volume and associated neuronal changes. In recent years an increasing number of studies analyzed the underlying changes at the level of MB neurons and synaptic complexes (microglomeruli, MG) in the MB calyces (Farris et al., 2001; Groh et al., 2004; Krofczik et al., 2008; Stieb et al., 2010, 2012; Dobrin & Fahrbach, 2012; Groh et al., 2012). Furthermore, Hourcade et al. (2010) showed that the formation of protein-synthesis dependent stable olfactory LTM leads to volume independent increase in the density of synaptic complexes in olfactory subregions of the MB-calyx. In the desert ant (*Cataglyphis fortis*) volume increase and changes in MG in visual input

regions (collar) of the MB calyx can be triggered by exposure to light, even in young ants, and long before the natural transition to outside foraging (Stieb et al., 2010, 2012).

In the honeybee, the transition from nursing to foraging is, among others, regulated by a rise in juvenile hormone (JH) and a corresponding decrease of vitellogenin (Vg) (reviewed in Bloch et al., 2002). JH is released from the paired corpora allata (Tobe, 1985) and upregulated in nurse bees prior to the transition to foraging (Jassim et al., 2000; Elekonich et al., 2001), and the yolk precursor protein Vg is produced in the fat body cells (Engels, 1974; Chapman, 1998). These two endocrine factors mutually suppress each other (Amdam & Omholt, 2003; Guidugli et al., 2005; Pinto et al., 2000), regulating division of labor (Marco Antonio et al., 2008; Nelson et al., 2007). High Vg titers in the hemolymph inhibit the onset of foraging (Nelson et al., 2007), whereas bees with a Vg knockdown showed a precocious onset of foraging associated with increased JH levels (Guidugli et al., 2005).

To shed light on the mechanisms underlying neuronal and behavioral plasticity associated with the age-related behavioral transition in the honeybee, we ask whether precocious sensory exposure to light leads to changes in the organization of MG in visual subregions of the MB calyx. We further address the question whether the JH/Vg feedback system affects changes in MG organization in the MB calyx. Finally, we test whether precocious light exposure interferes with the hormonal system.

Methods

Animals

European honeybees (*Apis mellifera carnica*) were collected from the institutional apiary close to the Biocenter of the University of Würzburg. Newly emerged bees from the same colony were marked in a climate chamber under red light, returned into the hive and recollected in the dark at different selected ages (approximately 4000 bees). To obtain winterbees, bees of unknown age were collected between 25th of January and 15th of February 2010 directly from a central frame of the winter cluster inside the hive.

For the knockdown experiments, freshly emerged bees (*Apis mellifera carnica*) from three different colonies at the apiary of the Arizona State University Polytechnic Campus (Gilbert AZ) were used.

Light exposure experiments

Winterbees (n=43), 1-day old bees (n=20) and 7-day old bees (n=20) were collected directly from the hive in darkness and divided in two groups. The first group was light exposed, and the second group kept in complete darkness, but otherwise treated the same. The two groups were kept in an incubator (32°C, 60% humidity), and fed with 40% sugar solution throughout the experiment. Light treatment took place in a dark climate chamber (30°C, 20% humidity) with an artificial light source (mercury arc

lamp, 125W, Exo-Terra Solar Glo) which emitted UV-light (UVA: 4,3 W/m^2; UVB: 0,05 W/m^2), visible-range light (69,0 W/m^2) and infrared radiation. To determine the natural light levels, light intensity was measured next to the hive in the shade (UVA: 15,71 W/m^2; UVB: 0,31 W/m2; PAR = photosynthetic active radiation at 400–700 nm: 186,27 W/m²), inside the hive close to the hive entrance (UVA: 0,0 W/m^2; UVB: 0,0 W/m^2; PAR: 0,0039 W/m²), and in the middle of the hive (UVA: 0,0 W/m^2; UVB: 0,0 W/m^2; PAR: 0,0018 W/m²) using an optometer (Gigahertz-Opik, model X1$_1$). To simulate repetitive exposures to light like during orientation flights and/or repetitive foraging trips over several days, bees collected at different ages were kept in small wooden boxes to be exposed to light in five intervals (45 min followed by 75 min of complete darkness) each day for three days in a row. Furthermore, 17-day old bees (n=8) that were confirmed foragers (natural foragers that had returned to the hive with pollen loads) were collected and transferred into constant dark conditions for three days (reversed foragers). Afterwards, they were compared with age-matched 20-day old natural foragers (n=8) from the same brood frame. To investigate the consequences of light exposure, bees were either further processed for immunolabeling to quantify MG in the MB calyx, or hemolymph was extracted for measurements of JH titers.

Immunocytochemistry

To visualize synaptic complexes in the MB calyces, immunochemistry as described by Groh et al. (2004) and Stieb et al. (2010, 2012) was performed. For brain dissections, bees were anaesthetized on ice, and after dissection, the brains were transferred immediately into fixative solution (4% formaldehyde in phosphate buffered saline, PBS) overnight at 4°C. On the following day brains were rinsed three times for 10 min in PBS and subsequently embedded in 5% agarose (Agarose II, no. 210-815, Amresco, Solon, OH). The brains were sectioned in 100 µm slices using a vibrating microtome (Leica VT 1000S, Nussloch, Germany). The slices were washed in PBS containing 0,2% Triton-X 100 (2x10 minutes each) prior to preincubation in PBS with 0,2% Triton-X 100 and 2% normal goat serum (NGS; Anova, 005-000-121) for one hour. To visualize microglomeruli (MG), slices were incubated with Alexa 488 Fluor Phalloidin, (Molecular Probes, A12379, 1:250 in PBS) and anti synapsin (1:50, SYNORF1, kindly provided by E. Buchner, University of Wuerzburg, Germany) in PBS with 2% NGS for three days at 4°C. Phalloidin binds specifically to f-actin in Kenyon cell dendrites (Frambach et al., 2004), and a monoclonal antibody to the *Drosophila* synaptic-vesicle-associated protein synapsin I marks the presynaptic boutons of MG (Klagges et al., 1996; Pasch et al., 2011). Afterwards, Alexa 568 goat@mouse (1:250 in PBS) was applied for two hours at room temperature and subsequently washed 5x in PBS (10 min each step). Sections were incubated in 60% Glycerol in PBS for 30 minutes before being mounted in 80% Glycerol on microscope slides.

Microscopy analyses and quantification of microglomeruli (MG)

To visualize brain structures, a confocal laser-scanning microscope (Leica TCS SP2, Wetzlar, Germany) was used. For the light exposure experiments, high magnification scans (objective: BL 63x1.4 OIL; with additional digital 2-fold zoom) of the lip and the collar regions of the two inner branches of the medial calyces were performed in both hemispheres using excitation wavelengths of 488 nm for visualization of f-actin-phallodin staining and 568 nm for anti-synapsin labeling. Single optical sections were scanned within a 100 µm thick brain slice. For this, a central plane was selected using the following anatomical landmarks: the upper unit of the central body, symmetrical planes of the MB calyces and the peduncles (Groh et al., 2004). Using the ellipse tool of the software FIJI (ImageJ 1.44c; National Institute of Health, USA), three circles with an area of 400 µm² were positioned in the dense region of the collar and two in the lip of the scanned images within the left and the right medial calyx of each animal, similar as introduced by Stieb et al. (2010). MG profiles within these circles were marked with the point tool when they included a synapsin profile encircled by an f-actin-phalloidin positive profile. MG profiles overlapping with the circle boarder were always included in the counts. The numbers of MG per circle were determined blind to the treatment, and the mean MG numbers per 400 µm² circle (MG / area) for each individual bee were calculated for the collar (mean of the six circles) and the lip (mean of the 4 circles).

dsRNAi knockdowns

In this experiment, single gene knockdown of ultraspiracle (*usp*) or vitellogenin (*vg*), and double gene knockdown of *vg/usp* were performed in newly emerged bees in order to dissect the function of both vg and JH in the neuronal maturation during division of labor. As JH is not directly accessible via gene knockdown, we approached the perturbation of JH by knockdown of one of its putative receptors, *usp* (Jones and Sharp, 1997; Miura et al., 2005; Riddiford, 2008) and by knockdown of vg. Previous studies showed that vg downregulation leads to a significant increase in JH (Guidugli et al., 2005; Nilsen et al., 2011; Wang et al., 2012) and advances the onset of foraging (Amdam et al., 2006). The *usp* knockdown was suggested to cause a compensatory increase of JH and the simultaneous downregulation of both vg and usp triggers an increase of JH that is significantly higher than in vg or usp single knockdowns (Wang et al., 2012, 2013). The double-stranded RNA (dsRNA) for the genes vitellogenin (*vg*) and ultraspiracle (*usp*) (single knockdowns) was prepared with a PCR using plasmid DNA and already established primers (Amdam et al., 2003; Barchuk et al., 2008; Wang et al., 2012). Green fluorescent protein (GFP) dsRNA was used as a control using AF097553 as a template, which does not exist in the honeybee. The DNA was further processed with the Qiaquick PCR purification kit (Quiagen, Frederick, MD, USA) and the dsRNA synthesized with RiboMAx Large Scale T7 RNA Production Systems (Promega, Madison, WI, USA). To purify the dsRNA purified phenol extraction was performed and the dsRNA diluted to 10µg/µl for the injections.

Newly emerged bees from three different colonies were pooled and randomly injected with dsRNA against *vg*, *usp*, *gfp* alone (single knockdowns), and against both *vg* and *usp* (double knockdown). Previous work has shown that injections of *vg* and *usp* trigger a specific RNA interference in honey bees resulting in the reduction of the corresponding mRNA (Guidugli et al., 2005; Amdam et al., 2003; Amdam et al., 2006; Barchuk et al., 2008; Wang et al., 2012). For the injections newly emerged bees were anaesthetized by cooling, and the dsRNA injected directly in the abdomen with a Hamilton syringe, following an established method for RNAi induction (Nelson et al., 2007; Wang et al., 2012, 2013). To increase the survival rate, injections were performed on two consecutive days. For the single knockdowns, the same amount of dsRNA was injected on days 1 and 2, whereas in the double knockdown, vg was injected on the first day and usp was injected on the second day which have shown the most effective way for double gene knockdown of vg and usp (Wang et al., 2012, 2013). Each treatment was marked with a color-coded dot of enamel paint (Testors Corporation, Rockford, IL, USA) on the bees' thorax, and the marked bees were transferred back into the same hive. Once bees were 7days old they were collected out of the hive, and their brains were dissected either for immunostainings, the fat body extracted for analysis of the mRNA level, or submitted to a starvation experiment.

RNA extraction and cDNA synthesis

After thawing and homogenization in TRIzol reagent, RNA was extracted following the manufacturer's instructions. The quality and quantity of RNA were determined by spectrophotometry (Nanovue, GE Healthcare). DNase (RNase-free, DNase kit, Applied Biosystems, Bedford. MA, USA) was added to the total RNA extract to remove trace DNA contaminants, and 1 µg of such treated RNA was used for reverse transcription following an established method (Wang et al., 2009) using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Bedford. MA, USA).

Validation of RNAi phenotypes

Quantitative real-time PCR analyses

First-strand cDNA was used for real-time quantitative PCR (RT-qPCR) assays. Sixteen samples were picked randomly from each treatment group for verifying *vg* and *usp* knockdown. Each biological sample ran in technical triplicates on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems, Bedford. MA, USA) for measuring *vg* and *usp* transcript levels in comparison with those of the reference gene *actin* by means of the Delta-Delta Ct method (Wang et al., 2012). Studies have shown that *actin* is stably expressed during different life stages in honeybees (Lourenco et al., 2008; Reim et al., 2012). By monitoring negative control samples (without reverse transcriptase) and melting curve analyses, we verified that the RT-qPCR assays were not confounded by DNA contamination or primer dimers (Vandesompele et al., 2002).

Starvation experiment

To acquire and compare the knockdown phenotype with the previous described phenotype for *vg/usp* knockdown, a starvation experiment was performed as a relative measure of the metabolic reserves of the bees (Wang et al., 2012). Bees injected with dsRNA (*vg*, n=35; *usp*, n=49; *gfp*, n=53; *vg/usp,* n=31) were collected at the age of 7 days out of the hive and harnessed in metal tubes. To quantify the survival under starving conditions they were kept in an incubator with 34°C and 80% humidity. The numbers of dead bees were recorded every three hours.

JH measurements

Groups of bees that were kept in the dark, submitted to a light-exposure program or directly obtained from the hive (1-day old, n=10; 4-day old, n=13; 7-day old, n=10-12; 10-day old, n=12, 26-day old foragers, n= 7-11) were anaesthetized on ice and fixed on a wax dish. Between 4-10µl of hemolymph was extracted by puncturing the dorsal cuticle of the abdomen through intersegmental membranes with a thin glass capillary. For measuring JH titers, the hemolymph volumes as well as the weight was used for calculations. The extracted hemolymph was immediately frozen in liquid nitrogen.

The hemolymph of individual honeybees was dissolved in 20 µL 50% (v/v) methanol containing 5 ng of juvenile hormone III ethyl ester (JH III EE) as internal standard, which was prepared by transesterification of commercially available JH III. Samples were sonicated for 2 min, centrifuged for 10 min at 14.000 rpm, and the supernatant was directly analyzed using LC-MS/MS.LC-MS/MS analyses performed using a Waters Acquity ultra-high-performance liquid chromatography system coupled to a Waters Micromass *Quattro Premier* triple quadrupole mass spectrometer (Milford, MA, USA) equipped with a electrospray interface (ESI). All aspects of system operation and data acquisition were controlled using MassLynx V 4.1 software. Chromatographic separation of JH III was carried out by reversed-phase chromatography using an Acquity BEH C18 column (50 x 2.1 mm, 1.7 µm particle size with a 5 x 2.1 mm guard column; Waters; Milford, MA, USA) with a solvent system consisting of water containing 0.1% formic acid (solvent A) and methanol (solvent B). A gradient elution was performed at a flow rate of 0.3 mL min^{-1} at a column temperature of 40°C from 60% to 100% B within 5 min, followed by 100% B for 2 min, and reconditioning at 60% B for 3 min. The injection volume was 10 µl.

Mass spectrometric conditions

For the mass-spectrometric detection, instrument parameters for an optimal ionization and fragmentation using collision-induced dissociation (CID) were optimized by flow injection of standard compound and internal standard. The electrospray source was operated in the positive electrospray mode (ESI^+) at 120°C and a capillary voltage of 2.75 kV. Nitrogen was used as desolvation and cone gas with flow rates of 800 L h^{-1} at 400°C and 50 L h^{-1}, respectively, the cone voltage (CV) was adjusted to 14 V. JH III was analyzed by multiple reaction monitoring (MRM) using Argon as collision gas at a

pressure of approximately 3 x 10^{-3} bar and a collision energy (CE) of 12 eV. JH III and JH III EE were identified by monitoring of four characteristic fragments for each compound with a dwell time of 25 ms per transition. Quantification was performed by integration of the area under one specific MRM chromatogram for JH III (m/z 267 > 147) and JH III EE (281 > 147).

Results

Changes in microglomeruli numbers after light exposure

To investigate the influence of light exposure on structural synaptic plasticity in the mushroom bodies, MG numbers in MB calyces of honeybees that were either exposed to light (4-day old: n=10, 10-day old: n= 10, 20-day old foragers: n=8) or kept in darkness (4-day old: n= 10, 10-day old: n=8, 20-day old reverted foragers: n=9) were analyzed. The number of MG was determined in three circular areas in the collar (Fig. 1) and in two circular areas in the lip in the left and in the right medial MB calyx as introduced by Groh et al. (2004) and Stieb et al. (2010).

A GLM Univariat Procedure ($p < 0.05$) was performed for the three circles in the collar region within the MB calyx. We found no significant difference between the circles throughout the data set. Therefore, we calculated the mean for the MG / area (number of MG per 400µm² circle) in the MB collar for the left and for the right medial calyx. Similarly, a paired t-test showed that the two circles in the MB calyx lip did not differ significantly, and we calculated the mean MG /area for the lip region for the left and the right calyx. Furthermore, paired t-tests did not show any difference in the relative density of number of MG / area in the collars of the left and right medial calyx branches. Therefore, only one mean for the collar and lip was calculated per 400µm² circle per individual bee. The numbers of MG in all experimental bees were normally distributed (One-Sample Kolmogorov-Smirnov Procedure, $p<0.05$).

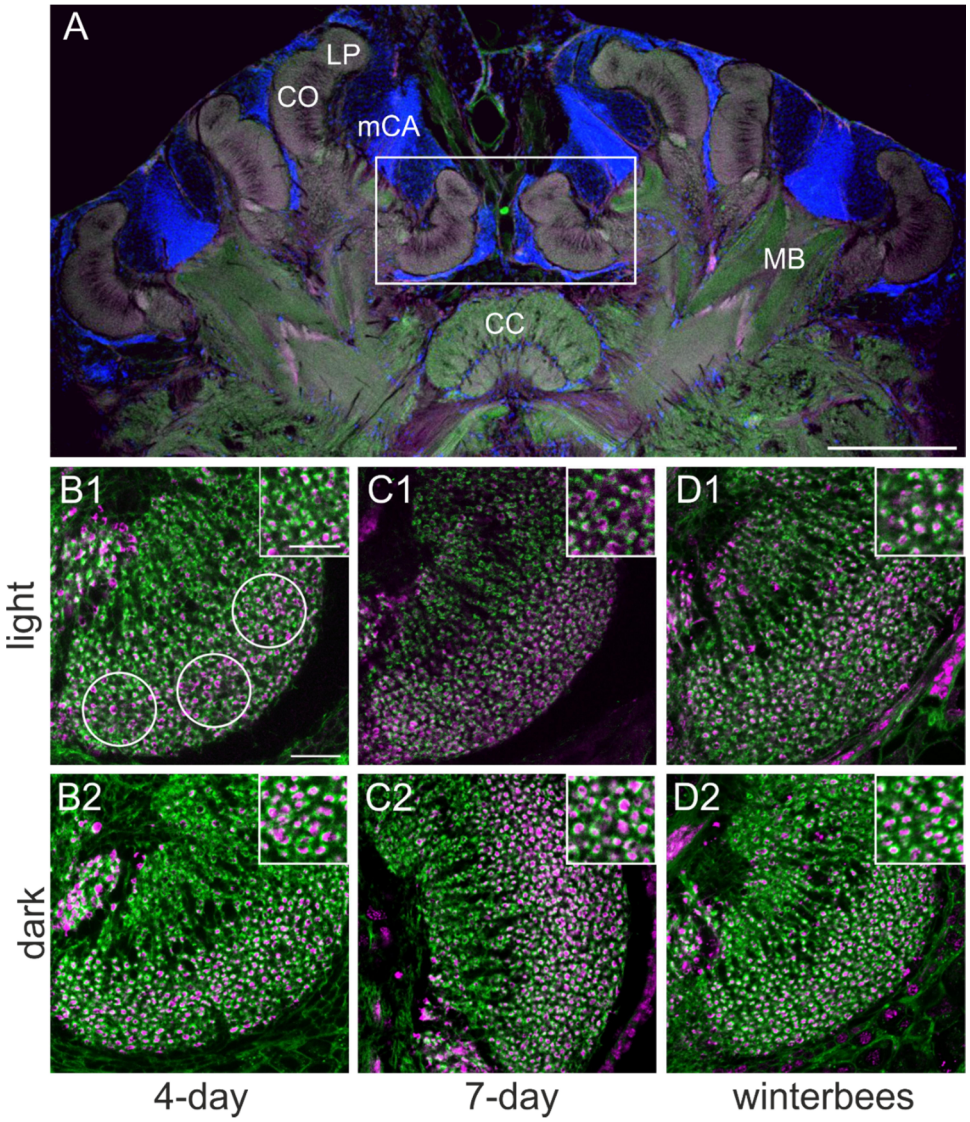

Figure1: Microglomeruli (MG) in the collar of the mushroom-body calyx
Anti-synapsin immunolabeling (red) combined with f-actin phalloidin staining (green) and Hoechst (blue) staining to label cell nuclei was used to visualize presynaptic and postsynaptic compartments of MG in the collar and lip regions of the mushroom bodies (MB) in light exposed and dark-kept bees of different ages. A: square indicates the calyces used for the analysis. mCA: medial calyx, CC: central complex, CO: collar, LP: lip B: 20-day old winterbees. Circles in B1 indicate the areas in which MG were counted. C: 4-day old bee D: 10-day old bee. B1, C1 and D1: Light exposed bees. B2, C2 + D2: Dark-kept bees. Scale bar: A: 200µm, B: 100µm inserts 10µm.

The number of MG / area in the olfactory innervated lip region did not differ across all age groups between the light exposed and the dark kept bees [Fig.2 (unpaired *t*-Test, 4 day old: p=0,101; 10 day old: p=0.382; foragers, p= 0.775: winterbees, p=0.536)]. In the visually innervated collar region, however, the number of MG / area was significantly altered between the light-exposed and the dark-kept group. In 4-day old bees, 10-day old bees, and in the winterbees, the number of MG / area in the collar region was significantly reduced after light exposure compared to the dark-kept control [Fig. 2 (unpaired *t*-test, 4 day old: p=0.000; 10 days old: p=0.000; winterbees: p=0.048)]. The reversed foragers, that were transferred back to the dark for three days compared with natural foragers that

were dissected directly after a foraging trip also showed a significant difference regarding the numbers of MG / area in the MB calyx collar. Here, the number of MG in the dark kept bees was increased compared to the untreated foragers [Fig. 2 (unpaired *t*-test, p=0.003)]. Across all tested age groups, light exposure resulted in a significant reduction of synaptic complexes (microglomeruli) in the visual subregions of the MB calyx (collar).

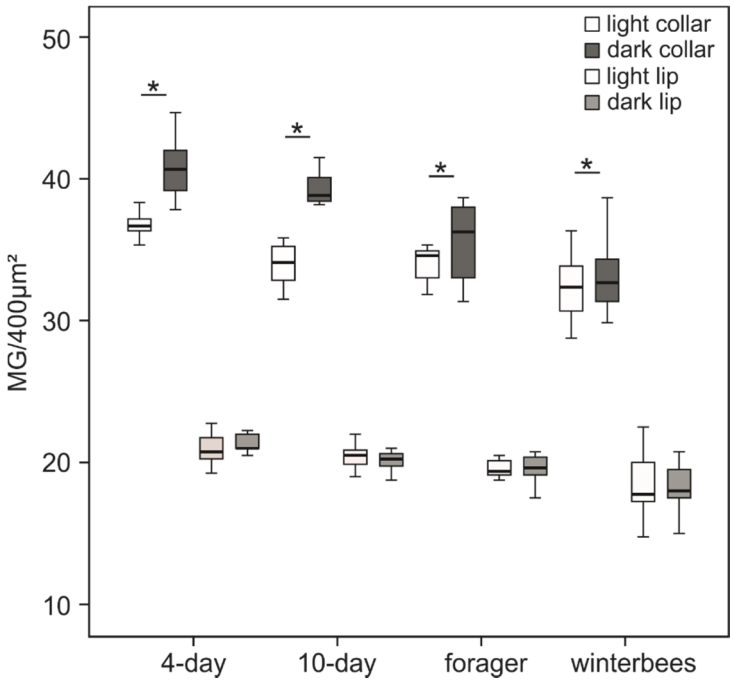

Figure 2: Influence of light exposure on the number of microglomeruli in the mushroom-body calyx
A: Bees (4-day old, n=10; 10-day old, n=10; winterbees, n=23; foragers, n=8) were exposed to light (5x45 min light pulses per day for 3 days) and the brains immunolabeled for microglomeruli (MG) detection in the lip and collar region of the mushroom-body calyx. The mean MG number of the 6 circles (400µm² each) in the collar per bee were compared to those in dark-kept bees (4-day old, n=10; 10-day old, n=10; winterbees, n=20; foragers, n=8). The visually innervated MB-calyx collar shows a significant reduction of MG after light exposure in 4-day old, 10-day old bees, and in winter bees. Natural foragers (17-day old) were transferred back to a dark environment after already experiencing several days of foraging. They showed an increase in the number of MG / area in the MB-calyx collar compared to natural foragers of the same age. **B**: In the olfactory innervated MB-calyx lip, the mean MG number (4 circles per bee, 400µm²) did not show a significant difference between all groups.

Validation of gene knockdowns for *vg* and *usp*

Six days after the dsRNA injection, RNA was isolated from fat tissue to validate the knockdown with real-time PCR. The transcript levels of *vg* and *usp* were measured in individual bees (n=16). The data for the *usp* and the *vg* knockdown was normally distributed (Kolmogorov-Smirnov-Test: *usp* knockdown: *vg*: p= 0,600, *usp*: p= 0,733, *gfp*: p=0,787, double: p=0,643;*vg* knockdown: *vg*: p=0,793, *usp*: p=0,649, *gfp*: p=0,921, *vg/usp*: p=0,830). In the *usp* and in the double knockdown (v*g/usp*), *usp* mRNA was significantly down-regulated compared to the *vg* and the *gfp* knockdowns [Fig. 3 (One way ANOVA with Tukey-HSD post hoc test, *vg-usp*: p= 0,000;*vg-gfp*: p=1,000;*vg*-double: p=0,002;*usp-gfp*:

p= 0,000, *usp*-double: p=0,062, *gfp*-*vg/usp*: p=0,020)]. The *vg* knockdown also showed a significant decrease of *vg* mRNA in the *vg* and the *vg/usp* knockdown compared to the *gfp* and the *usp* knockdown [Fig. 3 (One way ANOVA with Tukey-HSD-posthoc test: *vg*-*usp*: p= 0,000;*vg*-*gfp*: p=0,000;*vg*-double: p=0,066;*usp*-*gfp*: p= 0,501;*usp*- *vg/usp*: p=0,000;*gfp*- *vg/usp*: p=0,000)].

To compare the knockdown phenotype with previously described experiments the starvation resistance was examined. 7-day old worker honeybees that were injected with dsRNA when emerging were collected, mounted in single metal tubes, starved for three days, and the number of surviving bees was noted every 3 hours. Survival of the bees was significantly decreased in the double knockdown (*vg/usp*) compared to the control (*gfp*) (Supporting Information Fig. S1, Cox's F-Test: p=0,044). In the single knockdown of *vg* (Cox's F-Test: p= 0,468) and *usp* (Cox's F-Test: p=0,095), no significant difference occurred in survival rates compared to the double knockdown. There was no significant difference in the survival rates between bees in the two single knockdowns (*vg*-*usp*, p=0,500) as well as between *gfp* and the single knockdowns (Supporting Information Fig. S1, Cox's F-Test: *gfp*-*usp*: p=0,97; *gfp*-*vg*: p=0,584). Similar as already indicated by Wang et al. (2012) double-gene knockdowns may increase mortality by affecting metabolic physiology of the bees.

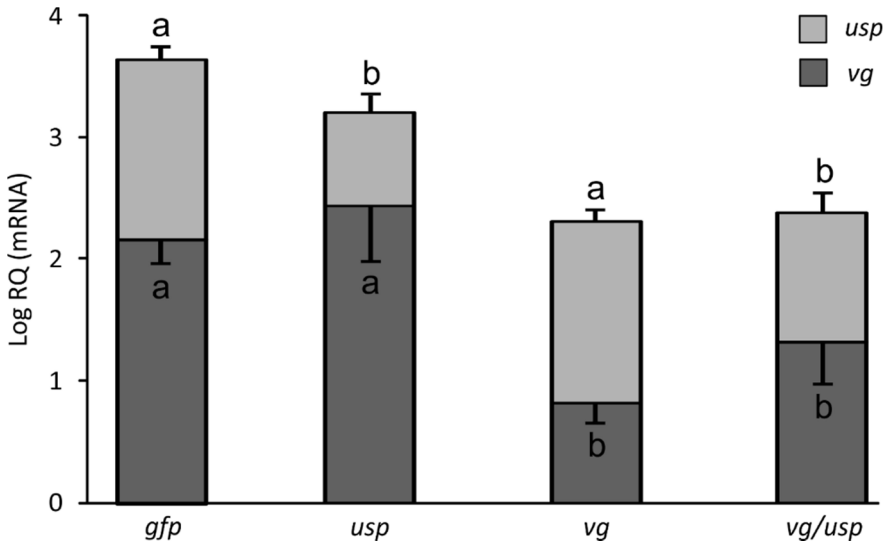

Figure 3: Verification of gene knockdown
Bees were injected with dsRNA against vitellogenin (*vg*), ultraspirale (*usp*), green fluorescent protein (*gfp*) (single knockdowns) and against both *vg* and *usp* (double knockdown, *vg*/*usp*). mRNA was extracted of fat body tissue of 7 day old bees. In the *vg* knockdown *vg* mRNA levels were reduces as well as *usp* in the *usp* knockdwown and both in the double knockdown (n=15-16).

Numbers of microglomeruli after gene knockdowns

To test whether the change in Vg/JH feedback system has a direct effect on the organization of MG in the MB calyx, we quantified the numbers of MG after down-regulation of vg and usp. The numbers of MG / area in the lip region was not different between the groups [Fig. 4 (One Way ANOVA with Tukey-

HSD post hoc test, vg-usp: p=0,961; vg-usp: p=0,746; vg-vg/usp: p= 0,950; usp-gfp: p=0,450; usp-vg/usp: p=0,749; gfp-vg/usp: p=0,973). Similarly in the collar region, we found no significant difference in the numbers of MG / area between the differently treated groups (One way ANOVA with Tukey HSD post hoc test, vg-usp: p=0,999; vg-gfp: p=0,971; vg-vg/usp: p=0,647; usp-vg/usp: p=0,581; gfp-vg/usp: p= 0,887). This indicates that under the tested conditions there was no significant direct effect of *vg*, *usp* and the interaction between *vg* and *usp* on reorganization of MG in the MB calyx.

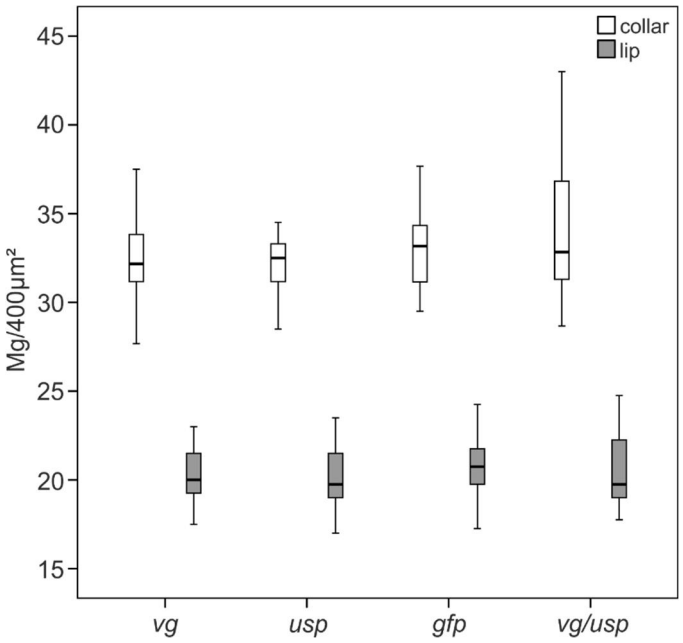

Figure 4: Influence of the knockdown of *vg*, *usp* and *gfp* on the number of microglomeruli in the mushroom-body calyx
To test a possible direct connection between the level of juvenile hormone (JH) and the numbers of microglomeruli (MG) during the nurse-forager transition, vitellogenin (*vg*), ultraspiracle (*usp*) or both (double knockdown, *vg/usp*) were downregulated using RNA interference. As a control, dsRNA against green fluorescent protein (*gfp*) was used. As high levels of *vg* inhibits JH production, downregulation of *vg* leads to an upregulation of JH.
In both the visually innervated collar region and in the olfactory innervated lip region in the mushroom body of 7-day old bees knockdown of *vg* and *usp* did not show any significant effect on the numbers of MG. Similarly in the double knockdown and in the control, no significant differences in the number of MG were detected.

Hemolymph JH titers in light exposed bees

Hemolymph (HL) was extracted from 1-day old, 4-day old, 7-day old, and 10-day old worker bees, as well as from foragers, and subjected to mass spectrometry to compare the JH titers in the HL. The bees were either exposed to light, kept in the dark, or caught directly in front of the hive as described above. All data were normally distributed (SPSS, Kolmogorov-Smirnov-Test, p< 0,05). The JH titer, in general, increased with the age of the bees; the untreated bees had the lowest amount of JH in the hemolymph at the age of one day, and the highest amount was found in the foragers (Fig. 5). The JH titers in the 10-day old bees did not differ significantly from those in experienced foragers. This may be due to the fact that those bees already had performed first orientation flights or even started early foraging (One

Way ANOVA with Tukey-HSD post hoc test: 1d-4d: p= 0,977; 1d-7d: p= 0,017; 1d-10d: p= 0,000, 1d-for: p= 0,000 ; 4d-7d: p=0,927; 4d-10d: p=0,020; 4d-for: p= 0,000; 7d-10d: p=1,000; 7d-for: p=0,131; 10d-for: p= 1,000).

After light exposure, the amount of JH in the 4-day and the 7-day old bees was significantly increased compared to the dark-kept and untreated bees (Fig. 6, One Way ANOVA with Tukey-HSD post hoc test,4-day old bees: light-dark: p=0,000, light-untreated p=0,000; 7-day old bees: light-dark: p=0,148 light-untreated: p=0,035). The JH titers in the dark and the untreated bees did not differ in both age groups (Fig. 6, One Way ANOVA with Tukey-HSD post hoc test, 4 days old bees: p=0,354; 7 days old bees: p=0,669). In the 10-day old group there was no significant difference in the JH titers between the differently treated groups (Fig. 6, One Way ANOVA with Tukey-HSD post hoc test, light-dark: p=0,753, light untreated: p=0,775, dark untreated: p=0,999). However, the variation in this age group was much higher than in the younger aged groups. This may also be due to the fact that bees at that age may have started first orientation flights as early as at 7 days of age, resulting in an inhomogeneous group of bees with different levels of light exposure and JH titers. Experienced foragers that were transferred back into the dark showed a significant decrease in their JH titers compared to foragers in which HL was extracted directly after a foraging trip (Fig. 6, unpaired t-Test: p=0,045). This gives further support to early experiments showing that foragers can be reversed to nurse bees (Rösch, 1930) including the associated changes in behavior and metabolism.

Bees that were exposed to light treatment also showed an increase of JH with age (Fig. 5, One Way ANOVA with Tukey-HSD, 4d-7d: p=0,062; 4d-10d: p= 0,000; 7d-10d: p=0,004). Even bees that were kept in the dark for three days to exclude any visual stimuli still showed a significant increase of JH with age between the 4-day old and 10-day old bees (Fig. 5, One Way ANOVA with Tukey post hoc test, 4d-6d: p=1,000; 4d-11d: p=0,009; 6d-11d p=0,340). The reversed foragers showed a strong trend for a decrease of JH titer in the HL compared to the 10-day old bees. This went all the way back to the level of JH levels typical for young nurse bees (4-day old and 7-day old bees) (One Way ANOVA with Tukey-HSD post hoc test, 4d-for: p=1,000; 6d-for: p=1,000; 11d-for: p= 0,092).

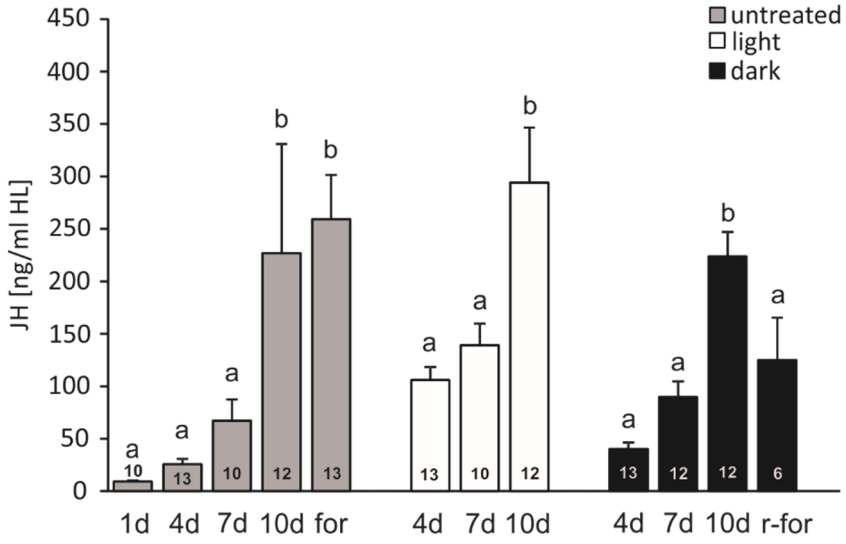

Figure 5: Age-dependent changes in juvenile hormone (JH) titers
The JH titers in the hemolymph of individual bees were measured in age-matched untreated bees, and bees that were either exposed to light or kept in the dark [1-day old (1d), 4-day old (4d), 7-day old (7d) worker bees and in 26-day old foragers (natural foragers: for, reverted foragers: r-for)] for 3 days using mass spectrometry. The JH levels increased with age, being significantly higher in 10-day old bees and foragers compared to 1-, 4- and 7-day old bees. The standard deviation was highest in the 10-day old bees.

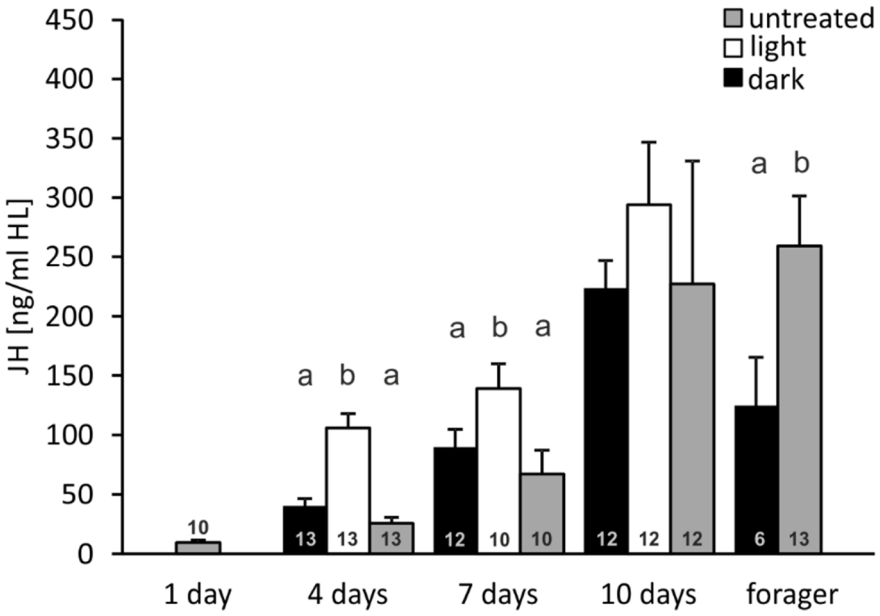

Figure 6: Juvenile hormone (JH) titers after light exposure
The JH hemolymph titers generally increased with the age of the bees. In untreated bees (untreated) the titer was lowest in newly emerged bees (1 day) and increased until it reached a level in 10-day old bees comparable to (26-day old) foragers. After light exposure, a significant increase in JH titers was visible in 4- and 7-day old bees compared to dark-kept bees as well as compared to untreated bees of the same age. 10-day old bees did not show lower levels of JH compared to the untreated and to the dark hold bees.

Discussion

Under natural conditions European honeybees (*Apis mellifera*) encounter light for the first time at high intensities when they are close to the hive entrance or when they leave the hive for orientation flights and start foraging (Seeley, 1982). To investigate neuronal and hormonal changes associated with this transition in sensory experiences, we used precocious light exposure in bees at different ages. Our results demonstrate that light exposure triggers significant reorganization of MG in visual subregions (collar) of the MB calyx in all age groups. These changes were triggered as early as adult day one indicating a high degree of plasticity in response to environmental stimuli. Similarly, 7-day old bees showed a decrease in MG densities after light exposure. Bees of this age may already start first orientation flights (Capaldi et al., 2000) suggesting that neuronal adaptations triggered by sensory exposure represent an important part of the transition from inside nurse duties (Farris et al., 2001). A similar role of sensory exposure was also shown in desert ants *Cataglyphis fortis* (Stieb et al., 2010, 2012). We chose winterbees as another age group to study the influence of light exposure during a period of hibernation inside the hive (Bodenheimer, 1937; Seeley and Visscher, 1985). Interestingly, winterbees showed a similar light-induced decrease in the MG density indicating a persistent neuronal plasticity in bees of up to 6 months of age. The fact that the olfactory innervated MB lip regions were not affected clearly demonstrates that MG reorganization is caused via sensory activation of the visual pathway rather than a general effect of increased plasticity after light exposure.

The decrease in numbers of MG / area after the nurse forager transition is associated with a significant outgrowth of KC dendrites and the reduction (pruning) of projection neuron boutons. This was shown by dendritic labeling and ultrastructural quantification of pre- and postsynaptic structures in honeybee nurses and foragers (Farris et al., 2001; Groh et al., 2012) and was further supported by MG analyses in the desert ants after light exposure (Stieb et al., 2010). Interestingly, (reversed) foragers that had already experienced several foraging trips showed an increase of MG after three days in darkness compared to age-matched foragers. Foragers, under natural conditions, have larger MB volumes than nurse bees (Withers et al., 1993; Durst et al., 1994; Farris et al., 2001). Fahrbach et al. (2003) showed that the MB volume did not change in reversed foragers. Our results indicate that MG densities may undergo reorganization in response to changing sensory environments independent of overall volume changes. Similarly, MG density increases after the formation of stable olfactory long-term memory without any volume changes of the MB calyx (Hourcade et al., 2010). To conclude, Rösch (1930) already showed the high behavioral flexibility of honeybee colonies to compensate for the loss of nurse bees by reversing foragers to nurses. The high degree of plasticity at the level of MG may enable the MB to “reset” flexibly to an initial state. Whether this is causal to a change in behavior requires further investigation.

What are the underlying molecular mechanisms of MG reorganization? Recently it was proposed that Rho GTPase signaling may mediate foraging-dependent changes in the mushroom body (Dobrin & Fahrbach, 2012). Furthermore, the plasticity associated calcium-calmodulin dependent proteinkinase II (CaMKII) was shown to be highly enriched in KC dendritic spines suggesting a potential role in the remarkable structural plasticity of KC dendrites (Pasch et al., 2011). The fact that light exposure leads to MG reorganization in the collar suggest that MG plasticity is triggered by the foraging experience itself and is not primarily due to age. This is also supported by previous work on MB plasticity in the honeybee (Withers et al., 1993; Farris et al., 2001; Whitfield et al., 2003; Ismail et al., 2006) and in the paper wasp *Polybia aequatorialis,* which shows dendritic plasticity in the MB due to social and sensory experience (Jones et al., 2009).

What is the role of hormonal systems in MG reorganization? JH was suggested as a main physiological driver of the nurse-forager transition (e.g. Velarde et al., 2009), and JH or its analog methoprene were shown to promote early foraging (Jaycox et al., 1974; Jaycox et al., 1976; Robinson, 1985). In the same direction, removal of the corpora allata resulted in flight impairment, decrease in metabolic rates (Sullivan et al., 2003), and late onset of foraging (Sullivan et al., 2000). Therefore, the question arises whether there is a direct link between the JH titer in the hemolymph and reorganization of MG in the MBs that both occur with the onset of foraging. Alternatively, triggering of the JH/Vg feedback system, in which a drop in *vg* gene expression causes an increase in JH (Guidugli et al., 2005) and an early onset of foraging (Nelson et al., 2007), could in itself induce MG plasticity, independent of the JH level per se. We tested these options in an experiment designed to trigger the JH/Vg feedback system by *vg* RNAi and to block the JH response system by *usp* RNAi. Our results clearly show that despite successful *vg* and *usp* gene knockdown there was no significant change in MG numbers. Combined with the result that removal of the corpora allata did not alter MB volume (Fahrbach et al. 2003) this indicates that the change in the JH/Vg feedback system (*vg* and double knockdown) as well as the JH response system (*usp* gene knockdown) has no direct influence on mechanisms controlling MB volume and MG reorganization under the conditions tested.

To find out whether precocious light exposure may trigger hormonal changes, we measured the JH titers after different treatments using mass spectrometry. The results in untreated bees show an increase of the JH level with age, with the lowest level in 1-day old bees, and the highest level in 26-day old foragers. Results from individual bees were consistent with previous findings obtained from pooled young nurse bees and foragers (Huang & Robinson, 1995; Jassim et al., 2000; Elekonich et al., 2001). Our method, therefore, proved as a useful tool to assess JH levels in individual bees. Interestingly, single bees within the 10-day old group showed high variations possibly due to their heterogeneous behavioral background. Some of the marked bees at this age did first orientation flights (data not shown). We therefore propose that the increase in variation of JH levels between 7- and 10-

day old bees may indicate an important point of time for a potential behavioral switch associated with an increase in JH.

After light exposure, 4- and 7-day old bees showed an increase in JH levels compared to age-matched bees or bees kept in complete darkness. Noticeably, the JH titers of the differently treated 4- and 7-day old bees compared to both older age groups. This suggests that light exposure alone does not increase JH to levels comparable to foragers at this age. In 10-day old bees, the difference between the groups was no longer statistically significant. The variations in the dark-held, light exposed and naturally kept bees were considerably higher and comparable to those observed in the untreated group. This indicates that additional factors affect JH increase in this age group.

In reverted foragers, MG showed a reversed plasticity. Our data indicates that this could also hold true for the hormonal system. Bees that revert from foraging to nursing were reported a drop in JH titer (Robinson et al., 1989; Robinson et al., 1992; Fahrbach et al., 2003). Our study suggests that lack of light input causes changes in both MG density in the collar and JH levels meaning that the change in sensory exposure at transitions in both directions triggers subsequent changes in MG organization and JH levels. Although these changes are likely to be important adaptations for the behavioral transition, they are not necessarily causal for the behavioral switch.

The molecular pathways upstream of light induced effects on MG reorganization and changes in JH levels still need further investigation. One potential candidate could be cGMP-dependent protein kinase (PKG), which is upregulated in foragers (Ben-Shahar et al., 2003). Interestingly, treatment with cGMP to increase PKG activity leads to precocious positive phototaxis (Ben-Shahar et al., 2003). This may provide a potential molecular link between changes in activity leading to light exposure. Future studies of light induced changes in gene expression have great potential to detect molecular pathways mediating plasticity in MB neuronal circuits and hormonal pathways promoting the behavioral transition.

In summary, our present findings support the hypothesis of a highly adaptable system in honeybee behavior and division of labor based on a high capability for adaptive responses to changing environmental conditions. We showed that light influences both the density of synaptic complexes in the MB and hemolymph JH levels suggesting that sensory exposure is an important parameter promoting behavioral adaptation to changing environments and social tasks.

Acknowledgments

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Manuscript II: Role of CaMKII in memory formation

CaMKII knockdown affects both early and late phases of olfactory long-term memory in the honeybee

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Abstract

Honeybees are able to solve complex learning tasks and memorize learned information for long time periods. The molecular mechanisms mediating long-term memory (LTM) in honeybees are, to a large part, still unknown. We approached this question by investigating the potential function of the calcium / calmodulin-dependent protein kinase II (CaMKII), an enzyme known as a "molecular memory switch" in vertebrates. CaMKII is able to switch to a calcium independent constitutively active state, providing a mechanism for a molecular memory, and has further been shown to play a critical role in structural synaptic plasticity. Using a combination of both knockdown via RNA interference and pharmacological manipulation, we disrupted CaMKII function during olfactory learning and memory formation. We found that learning, memory acquisition and mid-term memory were not influenced, but all manipulations consistently resulted in an impaired LTM. Both early LTM (24 hours after learning) and late LTM (72 hours after learning) were significantly disrupted indicating the necessity of CaMKII in two successive stages of LTM formation in the honeybee.

Keywords:

CaMKII, insect, olfactory learning, long-term memory, mushroom bodies

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Introduction

Higher order neuronal processes such as learning and memory are crucial for animals that need to be able to adjust their behavior to variable environmental conditions. By collecting, processing and storing information, they can use previous experiences to adapt their behavior according to specific needs. Honeybees are highly social and act as central place foragers to provide food supply to their colonies (Seeley and Visscher 1988; Menzel et al. 1999; Menzel and Giurfa 2001). To maximize their foraging efficiency, honeybees learn rewarding flowers, exploit new food sources and, most importantly, remember and share this information with other members of the hive (von Frisch 1967). To study learning and memory processes in the laboratory, learning assays – especially classical associative olfactory conditioning – have been well established (reviewed in Giurfa and Sandoz 2012). Whereas earlier attempts to unravel molecular and biochemical pathways associated with learning and memory in the honeybee were often based on pharmacological tools (e.g. Müller 1996; Müller 2000; Felsenberg et al. 2011), in recent years manipulations using genetic tools like RNA interference (RNAi) became more accessible (Farooqui et al. 2003; Müssig et al. 2010; El Hassani et al. 2012; Louis et al. 2012; Matsumoto et al. 2014). The possibilities for combination of several manipulative approaches and the availability of robust associative learning assays make the honeybee an excellent model organism to study the link between behavioral plasticity and its molecular bases.

One protein well known as a “memory switch” is the calcium / calmodulin-dependent Kinase II (CaMKII) (Lisman 1985). It has the ability to remain constitutively active even after the initial excitation of the neuron is no longer present (Miller and Kennedy 1986; Coultrap and Bayer 2012; Lisman et al. 2012; Malik et al. 2013). Due to its autophosphorylation after initial activation, the protein "switches" to a calcium independent constitutively active state, thereby providing a mechanism for molecular memory. In the mammalian hippocampus CaMKII is highly abundant in the CA1 region, a brain region that has become an important model system for understanding long-term LTP and long-term memory (LTM) formation (Kerchner et al. 2008). Here, CaMKII is necessary for the induction of LTP (Malinow et al. 1989; Giese et al. 1998; Lledo and Hjelmstad 1995), a process widely studied as one mechanism for learning and memory at the cellular and molecular level. Genetically manipulated mice with disrupted CaMKII function show deficits in learning tasks (Silva et al. 1992a; 1992b; Elgersma et al. 2002; Giese et al. 1998). Together with results from pharmacological blockade of LTP by application of CaMKII inhibitors (Malenka et al. 1989; Malinow et al. 1989) this suggests an important function of CaMKII in LTP and in memory formation.

It has been suggested that the hippocampus and the mushroom bodies (MB) may share a common ancestor and functional similarities (Nunes and Simões 2009; Tomer et al. 2010; Wolff and Strausfeld 2014). The MBs are paired structures in the insect brain that function as multisensory integration centers and are known to be involved in associative learning and memory processes in a variety of

insects, including the honeybee (e.g. Heisenberg et al. 1998; Menzel and Giurfa 2001; Heisenberg 2003; Gerber et al. 2004; Carcaud et al. 2009). Interestingly, in accordance with the results on hippocampal distribution and function, CaMKII is highly enriched in the MB of the adult insect brain (Kamikouchi et al. 2000; Takamatsu et al. 2003; Hodge et al. 2006; Pasch et al. 2011; Wolff and Strausfeld 2014). The first studies on the role of CaMKII in the behavior of insects have been performed in drosophila and showed the importance for CaMKII for learning and memory retention (Griffith et al. 1993, 1994; Joiner and Griffith 1999; Akalal et al. 2010; Malik et al. 2013). Recent studies in insects have indicated that pCaMKII is upregulated after learning (Lent et al. 2007) and inhibition of CaMKII leads to memory impairment (Matsumoto et al. 2014; Mizunami et al. 2014) which may indicate similar functions of CaMKII in memory formation in insects compared to vertebrates.

In the present study we analyzed the role of CaMKII in learning and memory in the honeybee *in vivo* by using RNAi to create a CaMKII knockdown phenotype. In a parallel approach and for comparability with other studies, we used pharmacological inhibition to disrupt CaMKII function. The minimal invasive manipulations were followed by olfactory conditioning and memory tests aimed to identify the potential contribution of CaMKII at various stages of memory formation in the honeybee brain.

Methods

Animals

For all experiments, European honeybees, *Apis mellifera carnica,* were taken from the apiary of the department of Behavioral Physiology and Sociobiology at the University of Würzburg. Active foragers identified by carrying a pollen load and caught in front of the hive entrance were used for all experiments. In all experimental procedures, the investigator was blind to the treatment identity of the bees.

Application of siRNA and pharmacological inhibitors

Honeybee foragers were caught the day before injections, immobilized in a refrigerator at 4°C, and harnessed in plastic holders. An acupuncture needle (Seirin, B2015) was used to poke a small hole through the median ocellus to insert a glass capillary (1B100F-3, WPI) pulled with a DMZ-Universal Puller (Zeitz Instruments). Using a microinjector (PV820 Pneumatic PicoPump, World Precision Instruments Inc.), 300nl diluted solution was injected trough the medial ocellar tract directly in the honeybee brain. Two siRNAs (siCaMKII and siGFP, 100µM), 2 CaMKII inhibitors (KN-62, 0.5 mM including 0.5% DMSO and KN-93, 0.5 / 1mM) and 2 controls (KN-92, 1mM and ringer solution including 0.5 % DMSO) were injected to manipulate the CaMKII.

To create a CaMKII knockdown phenotype, a specific siRNA against the enzyme (siCaMKII) with the sequence GAAUCGUGUGUCCUAUCAA (sense strand) and UUGAUAGGACACACGAUUC (antisense

strand) was designed (Eurofins). As a control a standardized siRNA vectored against GFP (siGFP) with the sequence GGCUACGUCCAGGAGCGCACC (sense strand) and GGUGCGCUCCUGGACGUAGCC (antisense strand) was used (Eurofins). The siRNAs were diluted in siMAX Universal Buffer (Eurofins) to reach a final concentration of 100 µM.

For pharmacological inhibition of CaMKII, the drugs KN-93 and KN-63 (Sigma-Aldrich Chemie Gmbh) were used. KN-93 inhibits the phosphorylation of target proteins of CaMKII as well as the auto phosphorylation of the enzyme itself (Gao et al. 2006). KN-62 is another CaMKII inhibitor that was shown to block enzyme activity by interfering with Calmodulin binding and to inhibit learning (Tokumitsu et al. 1990; Tan and Liang 1997). All chemicals were diluted in physiological ringer solution (130 mM $NaCl_2$ 5mMKCl 4mMMgCl 5mMCaCl2 15mMHepes, 25mM glucose, 160mM sucrose, pH 7.2). As KN-63 is not soluble in water, 0.5% dimethyl sulfoxide (DMSO) was added. As controls, an inactive form of KN-93 - KN-92 (Sigma-Aldrich Chemie Gmbh) or only ringer solution with 0.5% DMSO (ringer) were injected. And additional control was not injected at all (control).

Survival rates

To test a possible influence of CaMKII inhibitors and siRNA injections on the general life expectance of experimentally treated bees, the survival for each bee in the course of the conditioning process was recorded. Additionally, for each treatment group 50 bees were injected and kept in a wooden box (length: 10.5 cm, width: 6.5 cm, height: 4.5 cm) and their survival rate was observed for the following 5 days. The bees were able move freely in the boxes, and sugar solution (40% vol/vol sucrose) and water was always available.

Gustatory responsiveness score (GRS)

As a control gustatory responsiveness was tested using the proboscis extension response (PER) corresponding to the respective conditioning paradigm, 1 hour after the injection of inhibitors and 8 hours after the injection of siRNA. Each bee was tested by touching both antennae with a droplet of water followed by a concentration series of 1%, 1.6%, 2.5%, 4%, 6.3%, 10%, 16%, 25%, and 40% sucrose water solution with a 10 min interstimulus interval (adapted after: Scheiner et al. 2001). A PER was scored if a bee fully extended its proboscis after the antenna was touched with one of the liquids. The sum of PERs delivered a gustatory response score (GRS) ranging between 0 (no response) and 10 (response to all solutions including water).

Validation of CaMKII knockdown

RT-PCR

To validate the knockdown of CaMKII mRNA, 10 – 15 brains were dissected 2, 4, 6 and 24 hours after siRNA injection. The ocelli, the optical and the antennal lobes were excluded. The total RNA was extracted using the peqGOLD Total RNA Kit (Peqlab). 2µg RNA were reverse transcribed to cDNA using

QuantiTect Rev. Transcription Kit (Quiagen). Primers specific to the CaMKII (forward: CGTCATATGTTGCCAACTGGT, reverse: TTGAGCACGTTCAACAATGG) and to the housekeeping gene rp49, which is well established for adult honeybees (Laurenço et al. 2008) (forward: GACTGCATTCGAGCCAGAG, reverse: GGTGTACATGGGGATTCAGG (SIGMA), were used.

Amplifications were carried out with the real-time PCR system (Mastercycler realplex², Eppendorf) using KAPA SYBR FAST universal Master Mix (Peqlab) as per manufacturer's instructions. The samples were measured in technical triplicates. The obtained data was analyzed using the delta-delta CT method (Pfaffl 2001). Additional controls included negative control samples (without enzyme) and melting curves.

Quantitative western blotting

For the quantification of CaMKII protein levels 15 honeybees were injected with either siCaMKII or siGFP and subjected to western blot analysis. The experiment was repeated 3 times. Eight hours after the injection the bees were anesthetized on ice, the brains quickly dissected, transferred in Laemmli buffer and frozen in liquid nitrogen. The brain samples consisted of the central brain including the mushroom bodies, but excluded the optic and the antennal lobes. The brains were homogenized, heated for 5 minutes at 95° and subjected to polyacrylamid gel electrophoresis on 5 % stacking gel and 12.5 % separating gel (100 mA per gel, 1.5-2.5 h). After a short rinse in 0.1 % TBST (10mM Tris - HCl, pH 7.9, 150mM NaCl, and 0.1% Tween 20) the protein bands were blotted from the gel to a nitrocellulose membrane (230 mA, 75 minutes). The membrane was incubated for one hour in 5 % bovine serum albumin (BSA) in TBST and afterwards incubated with an antibody against pCaMKII (1:4000, sc-12886, Santa Cruz Biotechnology) and an antibody against actin (1:500, sc-1616, Santa Cruz Biotechnology). An antibody against the phosphorylated CaMKII (pCaMKII) was used as it shows constantly high proportions in immunostainings (Pasch et al. 2011) and correspond to CaMKII in situ hybridization studies (Kamikouchi et al. 2000). The membrane was rinsed in TBST (3 x 10 min) and incubated with the secondary antibodies IRDye 680@rabbit (1:20000, Licor biosciences) and IRDye 800@mouse (Licor biosciences, 1:20000) in 5% BSA in TBST for two hours at room temperature. After three rinses in TBST (10 minutes each) the membrane was analyzed with the Odyssey Infrared Imaging System (Licor biosciences). Prior to the experiment it was tested and ensured that both proteins were in linear range of the fluorescence values measured for the used protein concentrations. For each lane one value for the fluorescence intensity for the one actin band and one intensity for the four CaMKII bands was measured. As the four presumed isoforms (Pasch et al. 2011) were not easily distinguishable, only one value was measured per lane for all four pCaMKII bands together. For relative quantification, a pCaMKII/actin ration was calculated for each sample and the obtained relative pCaMKII levels normalized to the mean pCaMKII/actin ratio for each western blot experiment.

Conditioning paradigm

Adult honeybees (foragers) were always caught the day before conditioning and harnessed in plastic holders. The bees stayed in the holders for the duration of the experiments, and before and after handling they were kept in a climate chamber (25°C, 40% humidity). One hour after harnessing the bees were fed until saturation with 40% sugar solution. The next day they were fed with 30 µl (siRNA injection) or 15µl (KN-62 and KN-93 injection) sucrose solution and injected with the corresponding solutions 1 hour afterwards. Bees then were subjected to olfactory conditioning 8 hours after siRNA injection, 1 hour after KN-62 and KN-93 application (Lucchesi et al. 2010; Mizunami et al. 2014; Matsumoto et al. 2014) and additionally 18 hours after injection of KN-93 and KN-92.

The bees were trained by olfactory conditioning of the PER (Takeda 1961; Bitterman et al. 1983; Giurfa and Sandoz 2012). Previous studies have shown that three (and also five) learning trials are sufficient to induce long term memory lasting several days (Menzel 1999; Hourcade et al. 2009). We used a conditioning protocol where the bees learn to associate an odor (conditioned stimulus =CS) with a sucrose reward (unconditioned stimulus = US). Before starting the conditioning process each bee was tested for an intact PER by touching the antennae with 50% sucrose solution. Only bees that showed an intact PER before the experiment and at the end of the retention tests were used for the experiments. 1-nonanol and 2-hexanol were used as the CS in an alternating manner. 30 minutes before conditioning, bees were placed next to the conditioning setup to adapt to the surroundings. For each bee one trial lasted 30 seconds. After the bee was moved in front of an active air ventilation the first 13 seconds were used to familiarize the bee with the conditioning setup. Afterwards the CS was presented 4 seconds followed by an overlap of CS and US for 1 seconds and the presentation of the US alone for 2 seconds. During the first 3 seconds of CS presentation the occurrence of a PER was recorded. This procedure was repeated 5 times with an inter-trial interval of 10 minutes. To better compare the results with a pharmacological study that was published after the first experiments were performed (Matsumoto et al. 2014), we added 3 trial olfactory conditioning after KN-93 and KN-62 injection.

After conditioning the bees were returned to the climate chamber until memory retrieval was tested (1h, 24h or 72h after the learning trials). The learned odor (CS) and the NOd were presented for 3 seconds to assure a specific response to the learned CS and to rule out generalization effects. For every bee only one single post-training test (1h, 24h or 72h) was performed to exclude extinction of reconsolidation processes (Sandoz and Pham-Delègue 2004; Stollhoff et al. 2005; Stollhoff and Eisenhardt 2009; Plath et al. 2012). Bees that were tested for memory retention 24 and 72 hours after conditioning were fed 3 times a day until saturation and then were food deprived 5 hours before their retention test.

Data analyses

All statistical tests were performed with SPSS (SPSS Inc.). For western blot analyses, data were first tested for normality using the Shapiro Wilks test and subsequently analyzed with a Mann-Whitney-U-Test. RT-PCR statistical analyses were performed on ΔΔCt values, which were shown to be normally distributed, and a one way ANOVA including a Tukey post hoc test. GRS response was analyzed using the Kruskal Wallis Test.

For the learning acquisition of honeybees during olfactory conditioning, ANOVA for repeated measurements including Tukey HSD post hoc analysis was applied. In this case ANOVA evaluation is allowed, as the binary responses during PER conditioning imply equal cell frequencies and more than 40 degrees of freedom (Lunney 1970). To test whether there was a difference in memory retention between the different treatment groups after 1h, 24h and 72h, chi-square test was used. To compare the specific responses to the CS and those to the NOd, MC Nemars test was used. Differences in the survival rates were tested using the Kaplan-Meier test.

Results

CaMKII knockdown does not affect survival and sugar perception

To test the influence of siRNA and inhibitor injections via the ocellar tract on the general survival of the bees, the mortality rate during conditioning trials was recorded. Bees injected with the different inhibitors, siRNA and controls were observed over a period of 5 days. In the behavioral conditioning experiments no difference between all injected bees and the controls were apparent ($p = 0.122$, see table 1). On average 6.38 % of bees died during the experiment after 24 hours and 13.78 % after being harnessed in plastic tubes for 72 hours.

Similarly, bees that were kept in boxes for 5 days after the injection did not show a significant difference in the survival rates ($p = 0.578$, see tab. 1). Bees injected with pharmacological inhibitors, siRNA, and the control injected bees did not differ significantly regarding to their mortality rate over the observed period. Notably, most of the bees that did not survive died in the first few hours after the injection indicating that death in these cases may have been due to brain injuries. The majority of bees that survived the first day also survived for the rest of the 5-day period and did not show any apparent changes in behavior compared to untreated control bees.

Table 1 Mortality of bees after the injection of CaMKII siRNA and inhibitors
Bees were injected with siRNA against CaMKII (siCaMKII, control: siGFP) or with CaMKII inhibitors (KN-62, KN-93). Injection of KN-92 and ringer solution (ringer) and untreated bees (control) were used as control groups. The mortality rate during the olfactory conditioning experiments and after the bees were kept for 96 hours in boxes without behavioral treatment were recorded for the tested retention time point groups (1 hour, 24 hour and 72 hour). The percentage of surviving bees was recorded in each case.

Treatment	1h PER		24h PER		72h PER		96h box	
	n	survival [%]	n	survival [%]	n	survival [%]	n	survival [%]
Ringer	88	100	65	97.40	82	89.02	50	88
KN-62 (1h)	73	100	93	94.62	87	86.21	50	84
KN-93 (1h)	66	100	76	89.47	87	82.95	50	84
KN-93 (18h)	54	100	77	85.71	71	78.87	20	88
KN-92 (18h)	48	100	73	91.78	64	82.81	50	90
Control	62	100	60	93.33	61	78.69	50	92
siCaMKII	64	100	78	89.74	83	86.75	50	86
siGFP	77	100	77	90.91	80	85.00	50	84

Gustatory responses and the perception of sugar solution are vital for olfactory PER conditioning. Therefore, in an additional experiment the gustatory response score (GRS) was tested after the injection of inhibitors, siRNA and controls (adapted after Scheiner et al. 2001). All bees performed normal in perceiving the sugar concentrations, and the responses did not differ between any of the groups ($p = 0.915$, see fig.3). This proved that CaMKII effects after disrupting the enzyme are due to changes in the brain and not due to changes in the periphery.

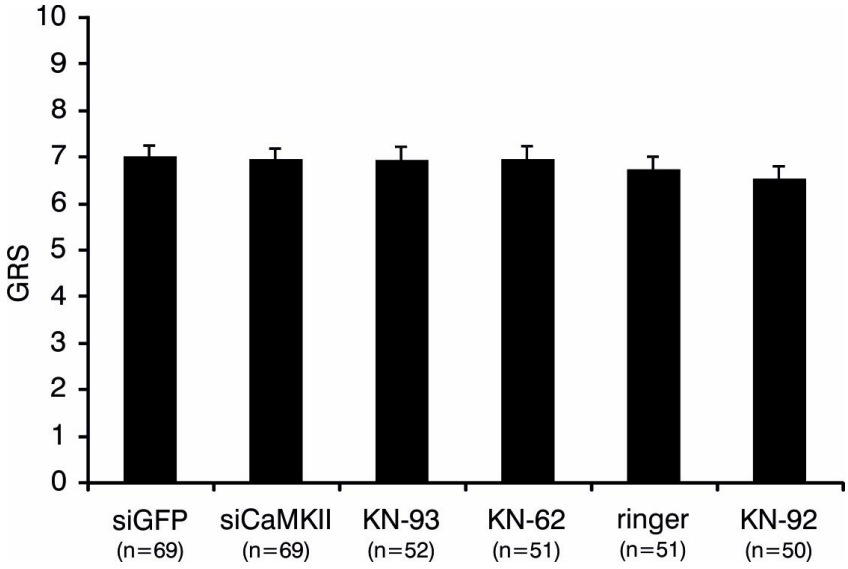

Figure 1: Sucrose responsiveness after injection of siRNA and CaMKII inhibitors
8 hours after the injection of siRNA against CaMKII (siCaMKII) and GFP (siGFP) and 1 hour after the injection of the CaMKII inhibitors KN-93, KN-62 and after injection of ringer only as a control, bees were tested for their gustatory response scores (GRS). Error bars represent the standard error.There was no significant difference between the different groups.

Specific knockdown reduces CaMKII mRNA and protein level

To verify a successful CaMKII knockdown, mRNA and protein levels after siRNA injection were examined. The mRNA levels had significantly decreased after siCaMKII injections reaching a minimum at 4 and 6 hours compared to the control (siGFP) injected bees (4h: siCaMKII-siGFP: $p = 0.002$; 6h: siCaMKII-siGFP: $p < 0.001$, see fig.1). A trend for a downregulation could be seen as early as 2 hours after injection (2h: siCaMKII-siGFP: $p = 0.359$, see fig.1), and 24 hours later the mRNA levels were back to the basal levels (24h: siCaMKII - siGFP: $p = 0.562$, see fig.1).

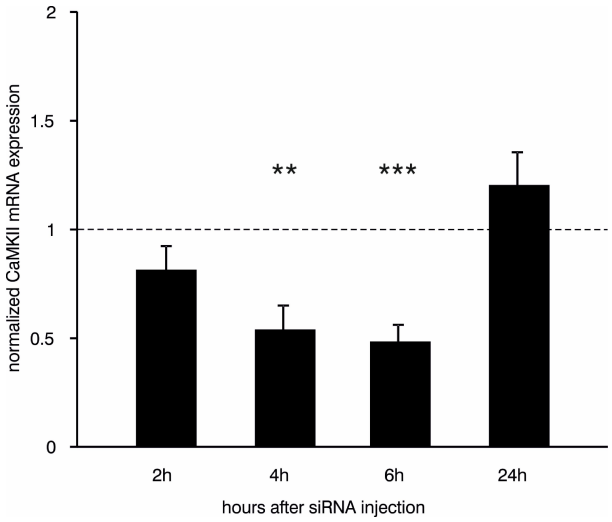

Fig. 2: Real-time quantitative PCR of CaMKII mRNA
Relative expression ratio of CaMKII mRNA in the brain after injection of siRNA against CaMKII. The expression ratio of CaMKII was normalized to RP49 mRNA and standardized to the control group (siGFP) at the different time points. Error bars represent the standard error. Asterisks indicate a significant reduction of CaMKII mRNA level in siCaMKII injected bees compared to siGFP injected bees.

Western blot analysis using an antibody against CaMKII matched the bands that can be seen using the antibody against pCaMKII (data not shown), but as the antibody against pCaMKII was more sensitive and showed a stronger signal it was used for the quantification.

Qualitative evaluations after 1 hour, 4 hours, 8 hours and 24 hours revealed a visible fluorescence reduction 8 hours after injection, whereas the other time points did not recognizably differ in the pCaMKII signal. Therefore, this time point (8 hours) was used for further analyses.

Quantitative analyses of the protein levels showed a reduction of the pCaMKII protein at 8 hours after siCaMKII injection compared to the control injected (siGFP) bees ($p < 0.001$, see fig.2). The protein amount, on average, was reduced by about 50%. As the four previously described pCaMKII bands (Pasch et al. 2011) were not clearly distinguishable, the fluorescence signals of all bands together were analyzed. Additional evaluation of the fluorescence level separated for the most prominent upper band and the three other bands together showed a similar downregulation effect (data not shown).

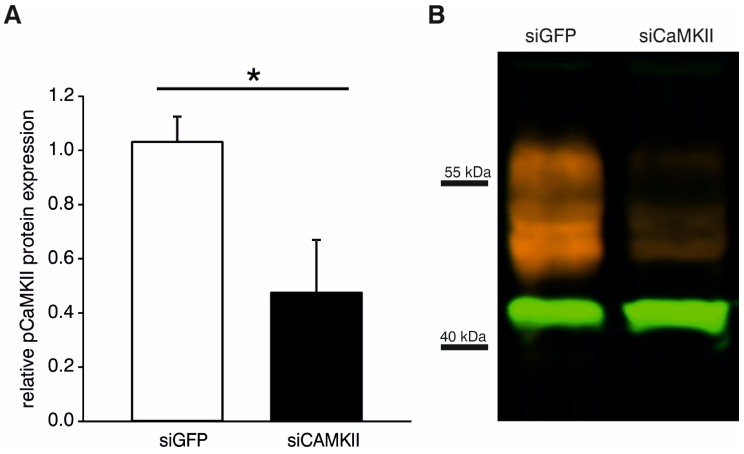

Fig. 3: Quantitative western blot analysis of the pCaMKII protein levels after siRNA injection
8 hours after the injection of siRNA against CaMKII (siCaMKII) and GFP (siGFP), protein levels in the central brain were measured using an antibody against the phosphorylated from of the CaMKII (pCaMKII). A: pCaMKII protein levels were standardized on actin protein level and normalized to siGFP injected control animals and show a decrease of protein level 8 hours after injection. Error bars represent the standard error. B: Western blot with pCaMKII visualized in red and actin in green using the odyssey imaging system

CaMKII knockdown as well as CaMKII inhibition leads to an intact memory acquisition and MTM, but disrupted LTM formation

To test if the CaMKII has an effect on memory formation, olfactory conditioning was performed after siRNA or inhibitor injection. As the learning curves of the different treatment groups (siCaMKII, siGFP, KN-92, KN-62, KN-93, control, ringer) did not differ between the 1h, 24h and 72h retention test time points, the learning acquisition data for each treatment group were pooled, and only one acquisition curve is shown for each treatment (One way Anova: siCaMKII, $p = 0.601$; siGFP, $p = 0.788$; control, $p = 0.738$; KN-92, $p = 0.458$; KN-93: $p = 0.122$. Tukey HSD Post hoc test also did not show significant differences between the different time points in any of the treatment groups).

CaMKII RNAi knockdown

After injection of siRNA the learning acquisition did not differ between siGFP and siCaMKII injected bees. Both groups showed typical learning curves (for comparison see Menzel 1999) with about 75 % of bees that learned to associate the odor with the sucrose reward after 5 learning trials ($p = 0.678$, see fig. 4A). However, the groups did differ in the retention tests. Whereas the mid-term memory (MTM) after 1h in siCaMKII injected bees was not significantly different from the control bees ($p = 0.416$, see fig. 4B), both early and late long-term memory (eLTM and lLTM) were significantly impaired in siCaMKII injected bees (24h: $p < 0.001$; 72h: $p < 0.001$, see fig. 4B). To make sure that a specific odor memory was attained, additionally to the conditioned odor (CS) a novel odor (NOd) was presented. The results showed that the specific response was always significantly higher than the unspecific response ($p = 0.015$).

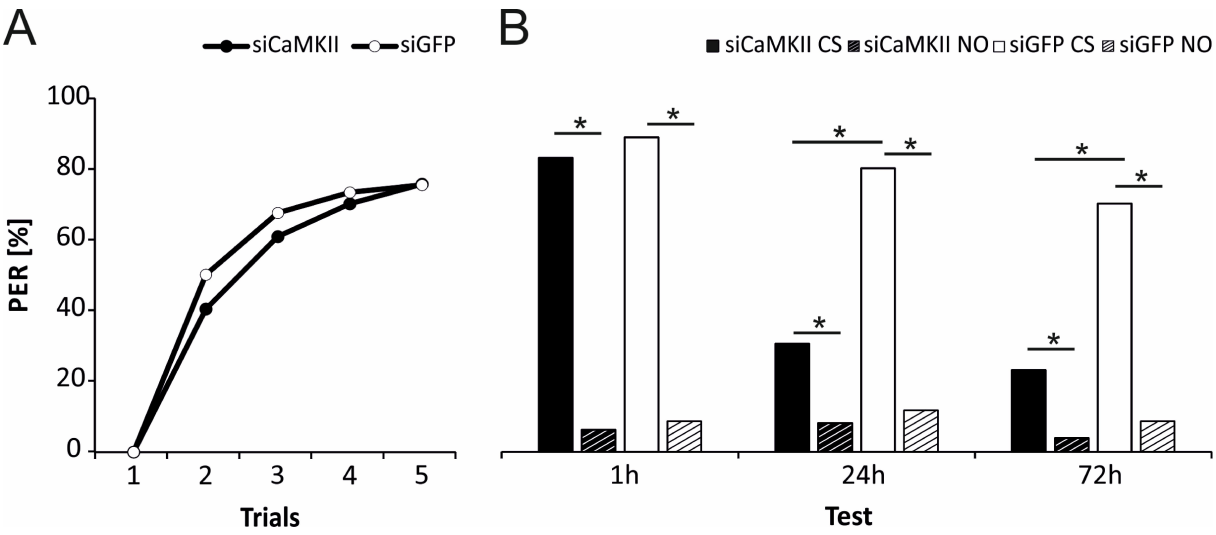

Fig. 4: Learning acquisition and memory retention after siRNA injection
Eight hours after injection of siRNA against CaMKII (siCaMKII) and against GFP as a control (siGFP) bees were conditioned in five trials to associate an odor (conditioned response=CS) with a sucrose reward. After different time points (1 hour, 24 hours and 72 hours) the responses to the CS and to a NOd were tested. The data for the acquisition was pooled for all subgroups, but every bee was tested only at one retention time (siCaMKII: 1h: n=63, 24h: n=66, 72h: n=67; siGFP: 1h: n=58, 24h: n=68, 72h: n=64).

CaMKII pharmacological inhibition

The CaMKII inhibitor KN-93 was injected via the ocellar tract to inhibit CaMKII. As a control, the inactive analogue KN-92 was injected and, additionally, an untreated control (control) was used. 18 hours after injection the bees were subjected to five trial learning. All three groups showed a normal learning acquisition (KN-92 - KN-93: $p = 1.000$, KN-93 – control: $p = 0.999$, KN-92 – control: $p = 1.000$, see fig. 5C). Retention tests one hour after training did not show differences between the inhibition of CaMKII and the controls (KN-92 - KN-93: $p = 0.494$, KN-93 – control: $p = 0.966$ KN-92 – control: $p = 0.517$, see fig. 5D). But similar to the CaMKII knockdown using RNAi, in both cases both the early and late phase long-term memory was disrupted after injection of the inhibitors (24h: KN-92 - KN-93: $p < 0.001$, KN-93 – control: $p < 0.001$, KN-92 - control: $p= 0.765$; 72h: KN-92 - KN-93: $p = 0.016$, KN-93 – control: $p = 0.001$, KN-92 – control: $p= 0.459$, see fig. 5D).

In another experimental series, the two CaMKII inhibitors (KN-62 and KN-93) and ringer (with 0.5 % DMSO) as a control were injected in the same way as before, but followed by a 3 trial olfactory conditioning paradigm 1 hour post injection. Again, the learning acquisition did not differ in the three groups (ringer - KN-62: $p = 0.0843$, ringer - KN-93: $p = 0.744$, KN-62 - KN-93: $p = 0.996$, see fig. 5A). In all groups on average 75% of the bees were able to associate the odor with a sucrose reward after three learning trials. Comparable to the previous inhibitor (KN-93) and siRNA injections, mid-term memory (1 hour) was not affected (KN-63- ringer: $p = 0.622$, KN-92 - ringer: $p = 0.614$, see fig. 5B). However, both early and late long-term memory were significantly impaired after injection of both

inhibitors compared to the control (24 hours: KN-63 – ringer: $p < 0.001$, KN-92 - ringer: $p < 0.001$ and 72 hours: KN-63 - ringer: $p < 0.001$, KN-92 - ringer: $p < 0.001$, see fig. 5B). In comparison to the conditioning 18 hours after injection (see fig. 5D), the inhibitors showed a ~ 20% stronger effect on the memory impairment for the bees if conditioning was performed 1 hour after injection (see fig. 5 B).

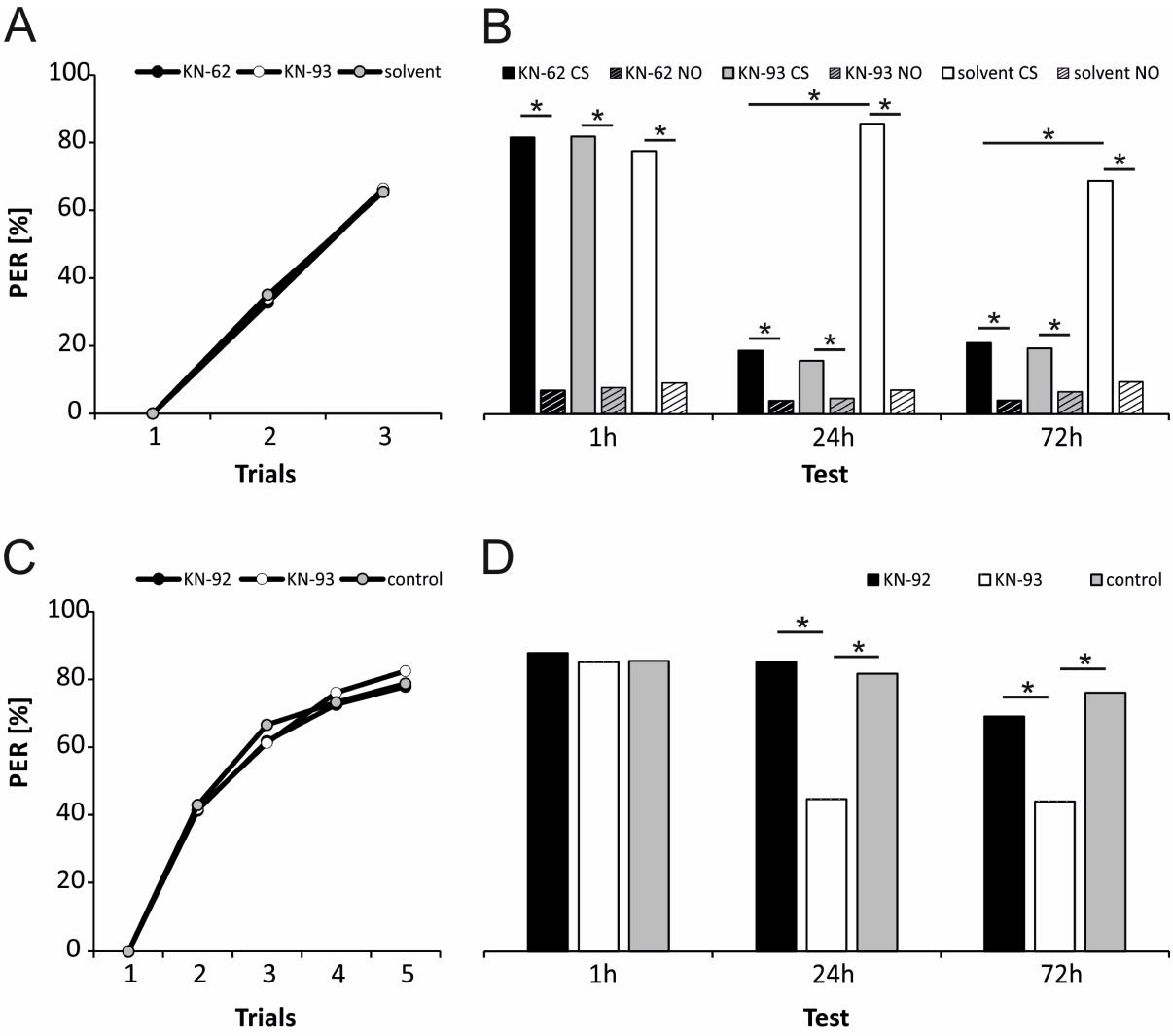

Fig.5: Learning acquisition and memory retention after injection of CaMKII inhibitors
A: The CaMKII inhibitors KN-62 and KN-93 were injected, and 1 hour later the bees subjected to a 3-trial olfactory conditioning to associate an odor (conditioned response=CS) with a sucrose reward using the proboscis extension response (PER). As a control ringer solution including 0.5% DMSO was injected and bees conditioned 1 hour afterwards. B: 1 hour, 24 hours and 72 hours later the bees were tested with the CS and a NOd (KN-62: 1h: n=70, 24h: n=87, 72h: n=73; KN-93: 1h: n=62, 24h: n=67, 72h: n=69; ringer: 1h: n=85, 24h: n=60, 72h: n=70). C: Bees that were injected with KN-93, its inactive form KN-92 and a control that was not injected at all were subjected to 5 - trial olfactory conditioning 18 hours after injection. D: Bees were tested 1 hour, 24 hours and 72 hours later (KN-93: 1h: n=54, 24h: n=66, 72h: n=56; KN-92: 1h: n=48, 24h: n=67, 72h: n=53; control: 1h: n=62, 24h: n=60, 72h: n=61).

Discussion

Using a combination of RNAi mediated knockdown and pharmacological inhibition, the results of this study strongly suggest that CaMKII plays an essential role in the formation of both early and late phases of LTM in the honeybee.

RNAi has been used more abundantly in recent years to study the function of proteins regarding to their role in honeybee behavior (e.g. Müssig et al. 2010; El Hassani et al. 2012; Louis et al. 2012; Leboulle et al. 2013). Especially in a social insect model organism like the honeybee, with transgenic manipulations not readily available, gene knockdown using RNAi is a powerful experimental approach. Pharmacological manipulations often have the disadvantage of unwanted effects on other proteins next to the original target. The CaMKII inhibitors KN-62 and KN-93, for example, were shown to act on L-type Ca^{2+} and voltage-gated K^{+} and Ca^{2+} channels (Li et al. 1992; Ledoux et al. 1999; Gao et al. 2006). For these reasons, and for reasons of comparability with other studies (see below), we chose a dual approach - using pharmacological inhibition and specific RNA interference in parallel.

We found that olfactory conditioning led to a normal acquisition phase and an intact MTM (1 hour retention) for all performed drug and RNAi manipulations of CaMKII. The memory phases in honeybees can be subdivided into short-term memory, lasting only seconds, MTM, lasting for several hours, eLTM, lasting 1-3 days and lLTM, that can be retrieved three or more days after conditioning (reviewed in Menzel 2012). In our experiments both eLTM (24h retention) and lLTM (72h retention) did differ significantly after drug (KN-62 and KN-93) and siCaMKII injection compared to the corresponding controls (ringer, siGFP, control). LTP and LTM are dependent on local protein translation from pre-existing mRNA pools in the postsynapse (reviewed in Steward and Schuman, 2001). In case of CaMKII it was shown that dendritic-specific translation is necessary for late LTP and to induce synaptic plasticity (Ouyang et al. 1999; Giovannini et al. 2001), which could explain the observed behavioral effect 8 hours after the knockdown although CaMKII the turnover rate in mice is $t_{1/2}$ = 3.01 days (Cohen et al. 2013). The fact that both - pharmacological and siRNA manipulations - showed the same behavioral phenotype strongly supports the role of CaMKII in early and late stages of LTM formation.

A recent study on the function of CaMKII in honeybees used KN-62 as an inhibitor and showed an impaired 72h retention, but in contrast to our present study, did not detect an effect after 24 hours (Matsumoto et al. 2014). We challenged these contradicting results by performing experiments using both inhibitors KN-62 and KN-93 together with the same learning assay as has been used in Matsumoto et al. (2014) (3 trial learning with same odor, novel odor) and confirmed our observation of a significant retention impairment both after 24 and 72 hours. This is in agreement with a related approach in crickets showing an intact MTM (1h retention) and an impaired eLTM (24h retention) after a three trial olfactory learning assay and inhibition via KN-93 and KN-62 (Mizunami et al. 2014). In the same line indirect and direct manipulations to decrease the amount of autophosphorylated CaMKII in the

mushroom body in *Drosophila* disrupted MTM as well as LTM (Malik et al. 2013). Different injection sites may also affect the time necessary for the drug to reach different brain regions, which could as well be important for different memory phases. In chicken, for example, it was postulated that different brain regions are responsible for short-term and mid-term memory and LTM. pCaMKII was upregulated in different brain centers during STM or LTM and inhibitor injection in different brain areas lead to the impairment of different memory phases (Zhao et al. 1999). Our study in the honeybee strongly indicates that CaMKII plays a role in different memory phases, which could be transmitted via different neuronal and molecular pathways and include either enzymatic active CaMKII or only structural functions of inactive CaMKII.

The fact that application of 2 inhibitors and specific RNAi in our study combined with equivalent observations in crickets and in *Drosophila*, we assume that the divergence with the findings by Matsumoto et al. (2014) is very likely due to the injection method and resulting differences in degradation and internal concentrations. One major difference between the study and our present study was the drug injection site (thorax vs. medial ocellar tract). As in the study by Matsumoto et al. (2014) the inhibitor was injected in the thorax and olfactory conditioning started only one hour later, this may not have given the drug enough time to interfere with early LTM mechanisms.

In honeybees it was shown that knockdown of NMDA-receptors (NMDAR) in the MB also affected eLTM formation, but not lLTM (Müssig et al. 2010). The formation of the CaMKII–NMDAR complex plays a key role in LTP induction and learning (Lisman et al. 2012). Together with our results this suggests a function of CaMKII in honeybees for the earlier stages of LTM formation. Further support for a function of CaMKII in eLTM comes from the fact that CaMKII was shown to affect f-actin remodeling (Okamoto et al. 2007) and AMPA phosphorylation (Barria et al. 1997a; Barria et al. 1997b; Derkach et al. 1999; Lisman et al. 2002), both are eLTM related processes that do not depend on translation mechanisms. CaMKII protein in honeybees was shown to be colocalized with f-actin in the postsynaptic dendritic spines of subpopulation of Kenyon cells (KC) in the MB calyx (Pasch et al. 2011) giving further support to potential molecular interactions of CaMKII with the actin cytoskeleton affecting spine motility and shape.

CaMKII was also shown to play a role in short term synaptic plasticity in the presynapse. CaMKII knockout mice show a disrupted short term plasticity independent of the kinase activity, but rather due to its structural functions via interactions with synapsin (Chapman et al. 1995; Hinds et al. 2003). CaMKII may also function in presynaptic plasticity in honeybees. Immunolabeling of pCaMKII showed a strong immunoreactivity in the MB vertical lobe (Pasch et al. 2011), which represents the presynaptic regions of KC axons (Strausfeld 2002). It is known that GABAergic neurons form a feedback loop from the MB lobes back to the MB calyx on KC dendrites and projection neuron boutons (Grünewald 1999). This feedback circuit may play a role in transmitting CaMKII mediated changes at the KC output site

back to the MB calyx. This retrograde pathway could potentially play a role in KC presynaptic contributions to early stages of memory formation in the MB in honeybees.

On the other hand lLTM could be mediated by strengthening the postsynapse through interaction of CaMKII with AMPA and NMDA receptors. CaMKII can phosphorylate existing AMPA channels, binds to NMDAR to increase their conductance and mediates vesicle transport to include new AMPAR in the membrane, all leading to prolonged changes in synapses (reviewed in Lisman et al. 2002). In honeybees knockdown of NR1 (honeybee sub-unit homologue of NMDAR) also shows an impairment of eLTM (Müssig et al. 2010) and mRNA detection of NR1 shows the same distribution as CaMKII mRNA in the KC somata (Zannat et al. 2006; Kamikouchi et al. 2000) indicating a possible interaction of these two proteins in the honeybee as well.

The data presented in this study suggest that CaMKII is an important mediator of both early and late phases of long-term memory formation in the honeybee. Pharmacological and RNAi manipulation of CaMKII function consistently resulted in a significant impairment of both early and late phases of LTM. The fact that different stages of memory were affected after the inhibitor was injected into different hemispheres (Zhao et al. 1999), indicates the possibility that this kinase plays a role in the formation of distinct memory stages according to its localization, possibly via interaction with different pathways.

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Author contributions:

Study design: CS, WR, TSM. Data collection: CS, NK. Data analyses and evaluation: CS. Discussion of results and manuscript writing: CS, WR.

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General discussion

In this thesis I focused on neuronal and molecular mechanisms involved behavioral plasticity in the honeybee. The first chapter addresses the synaptic plasticity that occurs during the nurse – forager transition discussing the role of sensory input (light exposure) on the synaptic network in the MBs. As I could show that light input influences MG circuits and JH is a mediator of division of labor I looked for a possible correlation between JH and MG density and JH and light exposure in the second chapter of the discussion. The last part of the discussion addresses another process associated with synaptic plasticity in the brain - learning and memory formation. As the molecular mechanism underlying learning and memory formation in honeybees still remains largely unknown, I investigated the role of the "memory enzyme" CaMKII in memory formation.

Sensory input as a key factor for the neuronal and behavioral nurse – forager transition

Under "normal" conditions honeybees workers express age-related division of labor. Young nurse bees stay inside the hive for the first 3 weeks as nurses, performing tasks like caring for the brood or cleaning the combs (Winston 1987). Subsequently, they start to search for food outside the hive (foragers) causing the confrontation with a variety of new tasks and challenges. Especially visual input suddenly becomes more important in the outside world. Using light exposure experiments in age controlled animals I could confirm in this thesis that light input triggers changes in the synaptic network in the dense collar region (visual input region; Gronenberg 2001) in the MBs of the honeybee. Foraging experience has previously been shown to alter MB volume in general and particularly volume of the visually innervated collar region (Withers et al. 1993; Durst et al. 1994; Ismail et al. 2006; Groh et al. 2012; Muenz et al. 2015). Correspondingly, synaptic rewiring in MG circuits takes place during the behavioral maturation of nurses to foragers (Krofczik et al. 2008; Muenz et al. 2015). The results of this thesis show that one part of foraging experience – net light exposure – seems to be sufficient to trigger changes in the MG network comparable to those observed in the natural age – and experience – related maturation. In the olfactory innervated lip region (Kirschner et al. 2006) the light treatment never triggered any effect on the density of MG. This supports the assumption that the effect is not a consequence of the general treatment of the bees, but specific to changes in visual environment and restricted to the region processing visual stimuli. Artificial light exposure for 3 days induced a decrease in MG densities in 7 day old bees that resembled densities observed in natural experienced foragers (17 days old). Similar effects of light input on MG density were also found in desert ants. Dark reared ants that were exposed to light for 4 days showed a decrease in MG in comparison to controls (Stieb et al. 2010, 2012). Interestingly my experiments showed that newly enclosed (1 day old) bees and winterbees of unknown age (up to 6 months old) also responded to light input with plastic changes in

the MB MG, indicating a highly plastic system even in this extreme age groups. 1-day old bees naturally do not approach the hive entrance but rather perform cleaning duties inside the dark hive (Winston 1987). After light exposure they showed a reduction of MG densities, but compared to the 7-day old group the densities were slightly higher not quite reaching the level of natural foragers. The less pronounced effect in newly emerged bees could be explained by a theory Susan E. Fahrbach postulated in 1998. She assumed that the MB outgrowth includes 2 phases, whereby the first phase (1-2 weeks after worker emergence) is dominated by "experience – expectant" MB growth (reviewed in Fahrbach 2006), followed by the second phase which includes "experience-dependent growth" (Farris et al. 2001; Ismail et al. 2006). Based on this theory 1 day old bees could predominantly express experience-independent maturation of the brain due to an internal program and therefore be less sensitive to the light exposure. 7 day old bees in contrast already perform first orientation flights (Capaldi et al. 2000) and their brain could already have undergone this first maturation phase enabling them to fully respond to the sensory stimulation, which in turn would explain the stronger decrease of MG densities. Supporting this explanation, bees that are deprived of light and social interaction still show an initial MB growth (Fahrbach et al. 1998). Moreover, Muenz et al. (2015) showed a pronounced volume increase only in the first week after worker emergence and no simultaneous change in the overall number of MG in the MBs supporting the hypothesis of an experience independent initial component of KC dendritic growth.

Experienced foragers that were placed in a dark environment for 3 days showed an increase of MG. Although honeybees show an age related division of labor, foragers are able to switch back from to nurse duties according if the colony demography lacks nurse bees for various reasons (for review see Robinson 1992). This behavioral plasticity seems to be accompanied by reversal plastic processes in the MG network in the brain. As the volume of MB does not change in reverse foragers (Fahrbach et al. 2003), it can be concluded that MG density may react to changing sensory input without affecting the MB volume.

But what are possible mechanisms for the decrease of MG densities in the MBs?

As adult neurogenesis is not present in the honeybee brain (Fahrbach et al. 1995; Farris et al. 1999; Ganeshina et al. 2000), mechanisms underlying change the existing MG network are most likely to be responsible for the observed changes. MG consist of a prominent presynaptic PN bouton and several f-actin rich postsynaptic KC dendrites (e.g. Farris et al. 2001). Both parts could be modified in light-activated neurons in the collar by either presynaptic axonal pruning of visual PNs and / or postsynaptic dendritic outgrowth of KC dendrites. Evidence for synaptic pruning is provided by sensory manipulation in honeybees that was shown to reduce the number of MG in the lip accompanied by an increase in volume of the presynaptic bouton (Krofczik et al. 2008). Additionally desert ants show a an increase in presynaptic synapsin labeling and decrease of MG after light exposure, further pointing to

pruning as a presynaptic mechanism to strengthen visual activated neuronal pathways (Stieb et al. 2012). On the postsynaptic side, KC dendritic spine outgrowth could lead increase the strength of MG synaptic transmission. KC dendrites were shown to express a bigger spine head and accelerate with foraging experience (Coss et al. 1980; Farris et al. 2001) and an increased number of postsynaptic contacts between presynaptic boutons and KC dendrites (Groh et al. 2012) in honeybees. Furthermore enrichment of tubulin fibers in KCs during maturation and after light exposure could explain the MB volume increase during maturation (Stieb et al. 2010; Muenz et al. 2015). Dendritic outgrowth and pruning due to visual experience in adult animals is also present in the visual system of vertebrates. For example Marik and collegues (2010) could show that sensory experience induces pruning and outgrowth of visual circuits in the visual system of mice in the matter of hours.

The molecular mechanisms underlying the changes in the synaptic circuit are still largely unknown, but in recent years several candidates have emerged. Genes that could play a role in the described synaptic reorganization and are upregulated in experienced foragers are for example insulin/insulin-like signaling (IIS) and *foraging* (Ben-Shahar et al. 2002; Ament et al. 2008). *Foraging* gene expression was shown to be accumulated in the MBs and correlates with the nurse – forager transition (Ben-Shahar et al. 2002; Thamm and Scheiner 2014). Another molecular link to the restructuring of the dendritic network might be provided by Rho GTPase. Rho GTPase activity is coupled to foraging experience and could mediate spine morphology due to its interactions with the f-actin cytoskeleton (Dobrin and Fahrbach 2012). Activation of muscarinic cholinergic receptors by neurotransmitters results in mushroom body volume increase and dendritic branching as observed in foragers indicating the involvement of these receptors in structural plasticity in the MBs (Dobrin et al. 2011; Ismail et al. 2006). Synapse pruning and stabilization are thought to involve LTP (e.g. Welberg 2014) and the in the MB highly abundant CaMKII (Pasch et al. 2011; Kamikouchi et al. 2000) was shown to be necessary for LTP induction (reviewed in Lisman et al. 2002). Therefore CaMKII could also be a factor triggering or influencing pathways that lead to the reorganization of synaptic network.

Finally to sum this up, the results of this thesis support the theory that sensory input resembling experience contribute to neuronal plasticity processes in the MB that accompany the nurse – forager transition. Furthermore the data supports the theory that both, a possibly internal experience-independent program as well as external experience-dependent stimuli contribute to synaptic reorganization in the MBs. The observed plasticity in newly emerged and overwintering old bees highlights the amazing adaptability of honeybees not only in behavior but also in the brain and draws attention to first week of adult life for future research.

Juvenile hormone - a potential mediator for the onset of foraging

The behavioral transition from nurse to forager is strongly associated with rising juvenile hormonal levels in the honeybee (for review see Robinson 1992). As changes in the synaptic circuits in the MBs occur coincidently, we considered a possible connection between the endocrine system and neuronal plasticity. To investigate this connection I used a method established by Amdam and colleagues (2003) using intra-abdominal injections of dsRNA to knockdown the genes JHs negative regulator *vg* and *usp* to disrupt the JH response system. The Knockdown of *vg* and *usp* causes an increase in JH level in the honeybee hemolymph, with the highest JH titer in a simultaneous knockdown of both genes (Guidugli et al. 2005; Amdam et al. 2007; Wang et al. 2012). Despite the successful knockdwon of both genes there were no corresponding changes in the MG density in the MBs. In line with these results, removal of the corpora allata did not alter MB volume (Fahrbach et al. 2003), indicating no direct effect of JH on synaptic restructuring. Even though the results did not show alterations of the MG network with our experimental approach, KCs in the MB do express receptors that are involved in JH signaling (Velarde et al. 2006) and could potentially be responsive to endocrine signaling. Ament and colleagues (2010) proposed that JH and insulin may interact with transcription factors to regulate gene expression in KCs, leading to the distinct gene expression profiles observed in nurses and foragers (Whitfield et al. 2003, 2006). Therefore we cannot exclude effects of JH on synaptic maturation in the MB, but at least with our experimental setting the increased JH level was not sufficient to trigger significant observable structural changes in the MG network.

As the results show that light exposure triggered a pronounced effect on synaptic circuits in the MB, the question for a possible effect of light on the endocrine system ascended. Therefore, we established a method to measure JH levels of individual bees, to be able to consider each bee's behavioral background. In untreated control groups a general increase of JH occurred with age. Correlating to previous work, JH levels in the hemolymph were lowest in 1 day old bees and highest in 26 – day old foragers (Huang and Robinson 1995; Jassim et al. 2000; Elekonich et al. 2001).

In 4 - and 7 -day old bees light exposure did indeed trigger an increase in JH level compared to controls that were kept in the dark. In the group of 10 – day old bees only a slight increase in JH level could be shown to be associated to light exposure even though the general variance in this age group was very high. The high variations in JH level could probably be due to the different behavioral background of individual bees and their sensory experience, which could mask an effect of light exposure on JH levels. Consequently, the JH level of light exposed 10 – day old bees was in the same range as JH levels in foragers. Accordingly an increase of JH in untreated bees was shown before they started foraging and did not differ in foragers and more experienced foragers (Jassim et al. 2000; Elekonich et al. 2001). We conclude that this age could be an important turning point for the behavioral switch from nurse to forager as it also coincides with the time point where experience-independent MB growth shifts to an

experience – dependent phase (Fahrbach 1998). It seems that high JH titers are needed prior to foraging. As older bees perform tasks closer to the hive entrance and thereby are more prone to be exposed to light, light could be one factor enhancing changes in JH titer before foraging. Notably bees that revert from foraging to nursing showed a drop to JH levels to the range of nurses, consistent with previous observations (Robinson et al. 1989, 1992; Fahrbach et al. 2003). A possible candidate in the molecular mechanisms leading to light induced changes in the endocrine system could be the cGMP-dependent protein kinase (PKG). This enzyme is upregulated in foragers (Ben-Shahar et al. 2002; Thamm and Scheiner 2014) and linked to age and phenotype which in turn is related to changes in phototaxis behavior (Ben-Shahar 2003). Interestingly *vg* / *usp* knockdown decreased the amount of PKG mRNA and one PKG isoform is expressed in the corpora allata further supporting a possible direct or indirect interaction of PKG with the endocrine system (Wang et al. 2012).

In summary the results in this thesis show that visual stimuli affect JH levels, but artificially increased JH levels were not sufficient to trigger structural changes in the synaptic network of the MB calyx. As light exposure both leads to pronounced changes in the MB and in JH levels in young bees as well as in "reversed" foragers, it can be concluded that sensory input is an important factor for behavioral adaptations during division of labor. However, further studies are needed to clarify the influence of JH on MB neuronal microcircuits. Furthermore, the interaction and causality of the different factors included in the nurse – forager transition need to be disentangled.

CaMKII is necessary for long term memory formation

MBs are not only important for behavioral maturation, they are also centers for learning and memory processes in the honeybee (for review see for example: Heisenberg 2003; Giurfa 2007). CaMKII is a protein known to contribute to synaptic plasticity (for review see Lisman 2002) and is highly abundant in the MBs (Drosophila: Takamatsu et al., 2003; Hodge et al., 2006; honeybee: Kamikouchi et al., 2000; Pasch et al. 2011; coackroach: Lent et al. 2007). To investigate the function of CaMKII in learning and memory formation in the honeybee I disrupted CaMKII function in the MBs by injecting two different inhibitors (KN-63 and KN-92) into the brain via the median ocellar tract. As both CaMKII inhibitors were shown to have side effects besides inhibiting CaMKII (Li et al. 1992; Ledoux et al. 1999; Gao et al. 2006), a specific CaMKII knockdown using siRNA was introduced for the first time in the honeybee. All three approaches to interfere with CaMKII function resulted in an intact memory acquisition and intact short and midterm memory (1 hour memory retention) after olfactory learning using the PER paradigm. However, both early LTM (24 hour memory retention) as well as late LTM (72 hour memory retention) retention was impaired for all CaMKII manipulations. These results are consistent with findings in crickets that also show intact memory retention 1 hour after conditioning but a disrupted 24 hours memory retention after inhibition of CaMKII using the same 2 inhibitors (Mizunami et al. 2014). In

insects first behavioral studies on CaMKII function were performed in *Drosophila* where CaMKII was also shown to be essential for memory formation (Griffith and Greenspan 1993; Griffith et al. 1994; Joiner and Griffith 1999; Akalal et al. 2010; Malik et al. 2013). Decreased pCaMKII levels after direct or indirect manipulation of CaMKII autophosphorylation in the MBs in *Drosophila* disrupted MTM as well as LTM (Malik et al. 2013). Behavioral data on the function of CaMKII contributing to learning besides *Drosophila* was first shown in the cockroach (Lent et al. 2007) and recently a first study indicated the importance of CaMKII for LTM formation in the honeybee (Matsumoto et al. 2014). In accordance to our data Matsumoto showed a disrupted 72 hours memory retention but contradictory an intact 24 hour memory retention after pharmaceutical CaMKII inhibition (Matsumoto et al. 2014). The present study used the same inhibitor (KN-62) and the same conditioning protocol, with the only difference being the site of drug injection. In the present study drugs were injected via the ocellar tract in the in contrast to injections in the thorax in Matsumoto et al. (2014). The injection in the thorax could result in different internal inhibitor concentrations and the incubation time may not be sufficient to reach all regions in brain compared to the injection via the ocellar tract, which brings all drugs much closer to the MBs. The injection site, therefore, could be crucial for affecting different memory phases. For example in chicken, different injection sites of CaMKII inhibitors in the brain caused deficits in different memory phases and pCaMKII was upregulated in different brain areas dependent on specific memory phases (Zhao et al. 1999). Evidence for memory traces in different KCs are also present in *Drosophila* where different KC types were shown to be important for different memory traces (Akalal et al. 2010; Malik et al. 2013). Confirming the behavioral data in this study using CaMKII inhibition, specific knockdown of CaMKII using RNA interference (RNAi) also showed an impairment in eLTM as well as lLTM. Disrupted LTM retention could be observed 8 hours after the injection of siRNA against the CaMKII, which might be surprising as the CaMKII turnover rate in mice is $t_{1/2}$ = 3.01 days (Cohen et al. 2013). But the activation of neurons does not only lead to the phosphorylation of existing CaMKII molecules, but also to a rapid translation of local CaMKII mRNA stored in dendrites (Ouyang et al. 1999; Giovannini et al. 2001).The dendritic machinery for translation may be essential for synaptic plasticity (reviewed in Steward and Schuman 2001) and protein synthesis of pre-existing mRNA pools in dendrites is necessary to induce LTP that persist for several hours (Bradshaw et al. 2003; Tsokas et al. 2005; Vickers et al. 2005). Therefore, we suggest that the CaMKII knockdown might deplete the local postsynaptic pools of CaMKII mRNA, thereby leading to the observed impaired memory-retention phenotype. Supporting this assumption, CaMKII mRNA is not only localized in cell bodies but also highly abundant in dendrites in the vertebrate hippocampus as well as in the honeybee MB (Walaas et al. 1988; Burgin et al. 1990; Kamikouchi et al. 2000; Pasch et al. 2011). RNAi has become an important genetic tool in studying connections between molecular mechanism and corresponding behavior in honeybees in recent years (e.g. Guidugli et al. 2005; Müssig et al. 2010; El Hassani et al. 2012; Louis et

al. 2012; Leboulle et al. 2013) and other studies also discovered a behavioral phenotype significant earlier than the protein turnover time of the corresponding protein (Müssig et al. 2010; El Hassani et al. 2012; Louis et al. 2012). The fact that pharmacological as well as siRNA manipulations in this experimental approach showed the same effect on memory formation strongly suggests a role of CaMKII in both eLTM and lLTM.

How can CaMKII influence different forms of memory and what could be molecular mechanisms?

CaMKII may mediate early forms of memory formation by influencing f-actin remodeling (Okamoto et al. 2007). LTP can induce changes in the dendritic spine morphology as fast as 60s after synaptic stimulation (Matsuzaki et al. 2004; Okamoto et al. 2004) and the activity – dependent enlargement of dendritic branches after LTP essential for structural plasticity of synapses requires CaMKII activity (Matsuzaki et al. 2004). In the honeybee CaMKII is colocalized with f-actin in the postsynaptic dendritic spines in specific subpopulation of KCs in the MB calyx (Pasch et al. 2011) providing the possibility for molecular interactions of CaMKII with actin to affect spine structure during memory formation.

A core function for CaMKII in strengthening synapses is by interaction with AMPA and NMDA receptors. CaMKII can for example phosphorylate existing AMPA channels, mediates vesicle transport to include new AMPAR in the membrane and bind to NMDAR to increase the conductance (reviewed in Lisman et al. 2002). The formation of a CaMKII / NMDAR complex is crucial for LTP induction and is blocked if the complex cannot be formed (Barria and Malinow 2005). Mutant mice with a point mutation in the CaMKII gene that prevents binding of CaMKII to NMDAR have deficits in memory recall 24 hours but not 1-2 h after training in the Morris water maze (Halt et al. 2012). Correspondingly knockdown of the honeybee NMDAR sub-unit homologue NR1 shows an impairment of eLTM (Müssig et al. 2010). Moreover, mRNA localization of NR1 shows the same distribution as CaMKII mRNA in the KC somata (Kamikouchi et al. 2000; Zannat et al. 2006) indicating a possible interaction of these two proteins in the honeybee, analog to vertebrates.

To summarize this part of my thesis, the results clearly show that both RNAi knockdown and pharmacological inhibition of CaMKII interrupts early as well as late phases of LTM in the honeybee. This strongly implies a role of CaMKII in the formation of LTM similar to vertebrates. As different stages of memory were affected, future studies are required to separate possible cell dependent memory traces and differential roles of CaMKII in temporal forms of memory. With several isoforms of CaMKII in the honeybee, it is possible that they may have different functions in different cells and should be analyzed in detail. The experimental approach used in this study creating a CaMKII knockdown phenotype is an important tool for future experiments clarifying functions of CaMKII in honeybees.

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