Mechanisms of visual memory formation in bees: About immediate early genes and synaptic plasticity

Mechanismen der visuellen Gedächtnisbildung bei Bienen: Über unmittelbar früh exprimierte Gene und synaptische Plastizität

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Investigating the physical basis of learning and memory

"… leads us into those most intimate fields of life, from which reason and intelligence originate."

Martin Lindauer, 1971

Communication among social bees

LIST OF CONTENTS

List of figures

List of tables

Summary

Animals form perceptual associations through processes of learning, and retain that information through mechanisms of memory. Honeybees and bumblebees are classic models for insect perception and learning, and despite their small brains with about one million neurons, they are organized in highly social colonies and possess an astonishing rich behavioral repertoire including navigation, communication and cognition. Honeybees are able to harvest hundreds of morphologically divergent flower types in a quick and efficient manner to gain nutrition and, back in the hive, communicate discovered food sources to nest mates. To accomplish such complex tasks, bees must be equipped with diverse sensory organs receptive to stimuli of different modalities and must be able to associatively learn and memorize the acquired information. Particularly color vision plays a prominent role, e.g. in navigation along landmarks and when bees identify inflorescences by their color signals. Once acquired, bees are known to retain visual information for days or even months. Numerous studies on visual perception and color vision have been conducted in the past decades and largely revealed the information processing pathways in the brain. In contrast, there are no data available on how the brain may change in the course of color learning experience and whether pathways differ for coarse and fine color learning. Although long-term memory (LTM) storage is assumed to generally include reorganization of the neuronal network, to date it is unclear where in the bee brain such changes occur in the course of color learning and whether visual memories are stored in one particular site or decentrally distributed over different brain domains. The present dissertation research aimed to dissect the visual memory trace in bees that is beyond mere stimulus processing and therefore two different approaches were elaborated: first, the application of immediate early genes (IEG) as genetic markers for neuronal activation to localize early processes underlying the formation of a stable LTM. Second, the analysis of late consequences of memory formation, including synaptic reorganization in central brain areas and dependencies of color discrimination complexity.

Immediate early genes (IEG) are a group of rapidly and transiently expressed genes that are induced by various types of cellular stimulation. A great number of different IEGs are routinely used as markers for the localization of neuronal activation in vertebrate brains. The present dissertation research was dedicated to establish this approach for application in bees, with focus on the candidate genes *Amjra* and *Amegr*, which are orthologous to the two

common vertebrate IEGs *c-jun* and *egr-1.* First the general requirement of gene transcription for visual LTM formation was proved. Bumblebees were trained in associative proboscis extension response (PER) conditioning to monochromatic light and subsequently injected with an inhibitor of gene transcription. Memory retention tests at different intervals revealed that gene transcription is not required for the formation of a mid-term memory, but for stable LTM. Next, the appliance of the candidate genes was validated. Honeybees were exposed to stimulation with either alarm pheromone or a light pulse, followed by qPCR analysis of gene expression. Both genes differed in their expression response to sensory exposure: *Amjra* was upregulated in all analyzed brain parts (antennal lobes, optic lobes and mushroom bodies, MB), independent from stimulus modality, suggesting the gene as a genetic marker for unspecific general arousal. In contrast, *Amegr* was not significantly affected by mere sensory exposure. Therefore, the relevance of associative learning on *Amegr* expression was assessed. Honeybees were trained in visual PER conditioning followed by a qPCR-based analysis of the expression of all three *Amegr* isoforms at different intervals after conditioning. No learning-dependent alteration of gene expression was observed. However, the presence of AmEgr protein in virtually all cerebral cell nuclei was validated by immunofluorescence staining. The most prominent immune-reactivity was detected in MB calyx neurons.

Analysis of task-dependent neuronal correlates underlying visual long-term memory was conducted in free-flying honeybees confronted with either absolute conditioning to one of two perceptually similar colors or differential conditioning with both colors. Subsequent presentation of the two colors in non-rewarded discrimination tests revealed that only bees trained with differential conditioning preferred the previously learned color. In contrast, bees of the absolute conditioning group chose randomly among color stimuli. To investigate whether the observed difference in memory acquisition is also reflected at the level of synaptic microcircuits, so called microglomeruli (MG), within the visual domains of the MB calyces, MG distribution was quantified by whole-mount immunostaining three days following conditioning. Although learning-dependent differences in neuroarchitecture were absent, a significant correlation between learning performance and MG density was observed.

Taken together, this dissertation research provides fundamental work on the potential use of IEGs as markers for neuronal activation and promotes future research approaches combining behaviorally relevant color learning tests in bees with examination of the neuroarchitecture to pave the way for unraveling the visual memory trace.

Zusammenfassung

Tiere erlangen Informationen über die Umwelt durch Lernprozesse und speichern diese Informationen durch Mechanismen der Gedächtnisbildung. Honigbienen und Hummeln stellen klassische Modellorganismen zur Untersuchung von sensorischer Perzeption und Lernvorgängen dar. Trotz ihres kleinen, lediglich etwa eine Millionen Nervenzellen umfassenden Gehirns sind diese hoch sozialen Bienen zu erstaunlichen Verhaltensleistungen fähig, welche komplexe Navigation, Kommunikation und Kognition einschließen. Auf der Suche nach Futterquellen navigieren Honigbienen über große Distanzen, ohne dabei die Lage ihres Nestes aus dem Gedächtnis zu verlieren. Außerdem sammeln sie hoch effizient Futter an zahlreichen morphologisch divergenten Blütentypen und kommunizieren neu erschlossene Futterstellen anderen Sammelbienen im Nest. Zur Bewältigung solch komplexer Aufgaben stehen Bienen diverse sensorische Organe zur Verfügung, womit sie Reize unterschiedlicher Modalitäten wahrnehmen und verarbeiten können. Außerdem sind sie zu assoziativem Lernen und dem Speichern und Abrufen von Informationen in der Lage. Insbesondere der Sehsinn spielt für Bienen eine große Rolle, wenn sie sich beispielsweise anhand von Landmarken orientieren oder farbige Blütensignale wahrnehmen. Einmal erlernte visuelle Informationen können mitunter über Tage und Monate hinweg gespeichert werden. Während die Aufnahme und Verarbeitung von Farbinformationen im Bienengehirn bereits gut untersucht wurde, ist über räumliche und zeitliche Abläufe der Speicherung solcher Informationen wenig bekannt. Mit der vorliegenden Arbeit wurde versucht, experimentellen Zugang zur visuellen Gedächtnisspur in Bienen zu bekommen. Die Bildung eines Langzeitgedächtnisses (LZG) geht im Allgemeinen mit Umstrukturierungsprozessen im neuronalen Netzwerk einher. Bislang ist es jedoch unklar, wo im Gehirn diese Veränderungen im Laufe des Farbenlernens stattfinden und ob Informationen in einem zentralen Bereich gespeichert oder dezentral über verschiedene Gehirndomänen verteilt werden. Unterschiedliche Verarbeitungsbahnen werden für das Erlernen grober und feiner Farbunterschiede vermutet. Mit der vorliegenden Arbeit wurden zwei Versuchsansätze gewählt, womit die Lage des visuellen Gedächtnisses untersucht werden sollte: Zum einen wurde die Eignung unmittelbar exprimierter Gene (immediate early genes, IEG) als genetische Marker für neuronale Aktivität untersucht, um damit frühe Prozesse der Bildung eines LZG lokalisieren zu können. Zum anderen wurden Spätfolgen der Bildung eines LZG auf die

Organisation synaptischer Netzwerke im zentralen Gehirn untersucht und der Einfluss der Komplexität einer Aufgabenstellung auf diese Organisation betrachtet.

IEGs sind eine Gruppe von Genen, die in Antwort auf zelluläre Stimulierung schnell und vorübergehend exprimiert werden. Zahlreiche IEGs werden bereits routinemäßig als Marker für neuronale Aktivierung im Gehirn von Vertebraten eingesetzt und mit der vorliegenden Arbeit sollten die Möglichkeiten evaluiert werden, diesen Ansatz auch in Bienen nutzbar zu machen. Hierzu wurde zunächst ermittelt, ob die Transkription von Genen überhaupt für die Ausbildung eines visuellen LZG von Nöten ist. Hummeln wurden mit Hilfe der Proboscis-Streckreaktion (PER) trainiert, monochromatisches Licht mit Zuckerbelohnung zu assoziieren. Nach erfolgtem Training wurde die Gentranskription pharmazeutisch gehemmt und die Gedächtnisleistung der Hummeln zu zwei Zeitpunkten ermittelt, die das Mittelzeitgedächtnis (MZG) bzw. LZG repräsentieren. Es zeigte sich, dass Gentranskription nicht für die Ausbildung des MZG, jedoch für die des LZG unabdingbar ist. Als nächstes wurden mögliche Kandidatengene validiert. Honigbienen wurden entweder mit Alarmpheromon oder einem Lichtimpuls stimuliert. Die Bienengehirne wurden anschließend seziert und mittels qPCR die Expression von *Amjra* und *Amegr* untersucht, zweier Gene, deren orthologe Vertreter *c-jun* bzw. *egr-1* gebräuchliche IEGs in Vertebraten darstellen. Während durch beide Reize die Expression von *Amjra* in allen Gehirnbereichen (Antenalloben, optische Loben und Pilzkörper) induziert wurde, konnten keine Veränderungen in der Expression von *Amegr* festgestellt werden. Daraufhin wurde überprüft, ob die Induktion von *Amegr* möglicherweise abhängig von assoziativen Lernvorgängen ist. Honigbienen wurden mittels PER visuell konditioniert, bevor die Pilzkörper zu verschiedenen Zeiten nach dem Training isoliert und mittels qPCR auf die Expression von *Amegr* Isoformen untersucht wurden. Hierbei konnte kein Lerneffekt auf die *Amegr*-Expression nachgewiesen werden.

Die Analyse Aufgaben-abhängiger neuronaler Korrelate, die der Bildung des visuellen LZG zugrunde liegen, wurde anhand frei-fliegender Honigbienen durchgeführt. Diese wurden entweder absolut konditioniert auf eine von zwei ähnlichen Farben, oder differentiell auf die Diskriminierung beider Farben. Bei der anschließenden unbelohnten Präsentation beider Farben bevorzugte nur die differentiell trainierte Gruppe die zuvor gelernte Farbe, während absolut konditionierte Bienen zufällig wählten. Um zu ermitteln, ob die beobachteten Unterschiede im Verhalten auch auf neuroanatomischer Ebene repräsentiert werden, wurden alle Bienen nach drei Tagen seziert und mittels Immunfärbung synaptische Komplexe, so

genannte Microglomeruli, im visuelle Informationen verarbeitenden Bereich der Pilzkörper quantifiziert. Der Vergleich zwischen den Versuchsgruppen legte keine signifikanten Unterschiede in der neuronalen Architektur offen, jedoch wurden mögliche Zusammenhänge zwischen Lernleistung und Microglomeruli-Dichte gefunden.

Die vorliegende Arbeit bietet grundlegende Ergebnisse zum Potential von IEGs als Marker neuronaler Aktivität und unterstreicht die Bedeutung integrativer Versuchsansätze, welche Verhaltensuntersuchungen mit der molekularen und histologischen Analyse des Nervensystems verbinden, um letztlich das visuelle Gedächtnis im Bienengehirn lokalisieren zu können.

Chapter I – General Introduction

Animal learning and memory

Learning is considered as an intended or unintended acquisition of knowledge or abilities based on experience that might lead to a modification of behavior to enable an organism to phenotypically respond to shifting environmental conditions (based on definition and discussion by Alloway 1972, Lachman 1997). Certain forms of learning can be found in quite different phyla of the animal kingdom, from nematodes (*Caenorhabditis elegans*: Rankin *et al.* 1990), molluscs (*Octopus*: Young 1961, *Aplysia*: Pinsker *et al.* 1969, discussed in Menzel 2012) and arthropods (honeybee: von Frisch 1914, cockroach: Sakura and Mizunami 2001) through to vertebrates (dog: Pavlov 1927, dove: Morse and Skinner 1958), including humans.

In all cases, learning is driven by experience and is facilitated by the structural plasticity of a precisely interconnected network of neuronal cells, like ganglia or brain structures. This plasticity mainly comprises changes in the strength, or even number of synapses (Kandel 2001, Holtmaat and Svoboda 2009, Ryan and Grant 2009). Synapses are the junctions between two nerve cells or between a nerve and another cell (e.g. muscle or gland cell), respectively, where information transmission from one axon to another cell occurs via chemical substances. Learning, therefore, results from changes in the effectiveness of the chemical signal transmission, and hence the strength of these junctions (Kandel 2001).

To recall learned information after a certain time, a memory must be established, which, in the case of long-term memory, goes beyond a strengthening of the preexisting synaptic connections, but also includes the complete degradation of existing synapses and the formation of new ones (Bailey and Chen 1988, Holtmaat and Svoboda 2009). In this way, the neuronal network undergoes dynamic structural changes. Long-lasting neural reorganization and synapse neoformation, which underlie persistent memory storage, seem to be common characteristics of neuronal networks in most animal groups, including mammals and insects (Bitterman 1975, Holtmaat and Svoboda 2009, Hourcade *et al.* 2010).

The Honeybee – A model for learning and memory

To gain insights into the widely unknown molecular and neuronal processes that facilitate (visual) learning and memory formation in invertebrates, experiments should ideally be

conducted with a model organism comprising a relatively low neuronal complexity. This was the reason, apart from the advantage of many gigantic, uniquely identifiable nerve cells, that brought Kandel and his collegues to conduct their seminal studies on the giant marine snail *Aplysia californica* (Frazier *et al.* 1967). Additionally, the experimental animal should exhibit complex cognitive performances and, importantly, the fundamental processes should show similarities to other taxa to be able to compare and potentially transfer possible findings. All these requirements are met by the honeybee (insects; Hymenoptera; Menzel and Müller 1996).

Honeybees (*Apis mellifera*) depict a qualified system for neuroethological studies and unraveling molecular and neuroanatomical coherencies of learning and memory. The honeybee brain is about 1mm³ in size and comprises solely approx. 960 000 neurons, which are in part individually identified and characterized (Menzel and Giurfa 2001). Nevertheless, it allows for an astonishing variety of behaviors and cognitive capabilities. Honeybee foragers can easily fly several kilometers from the nest to a suitable food source and handle hundreds of morphologically divergent flowers in a quick and efficient manner to gain nutrition (nectar and pollen). The foraging efficiency is optimized in bees by adaptive mechanisms underlying orientation and navigation. Bees can make use of landmarks (Cartwright and Collett 1983, Cheng *et al.* 1986) and learn the spatial relationship between nest and foraging site through the integration of information about solar altitude and sky polarization pattern (Menzel *et al.* 1990, Evangelista *et al.* 2014). Moreover, honeybees can measure flight distance by means of the optical flow, which is perceived through the compound eye (Srinivasan *et al.* 2000). Once returning to the hive, foragers communicate the direction and distance of a profitable food source to their nest mates by performing ritualized body movements, the so-called round and waggle dances, respectively (depending on the communicated distance; von Frisch 1965, Esch *et al.* 2001, Dyer 2002). Following nest mates learn the flower odor that is attached to the dancing bee and depart to search for the specific food source at the indicated location (von Frisch 1927, Barth 1985).

An essential element of the foraging behavior constitutes associative learning, whereby an association can be formed between an originally neutral stimulus and a reward. Odor, color and shape of a flower are learned when a bee perceives these stimuli just before she encounters food (nectar, pollen). This appetitive learning is a form of associative learning,

which is well known from behavioral studies in mammals (Bitterman 1975, Hammer and Menzel 1995).

Associative learning in bees, that is supposed to be an important factor in foraging success, is often considered a typically fast and robust process (Hammer and Menzel 1995, Raine and Chittka 2008). A single or few olfactory learning trials can lead to highly significant changes in behavior and response qualities of identified neurons (Mauelshagen 1993, Strube-Bloss *et al.* 2011). This kind of learning is even possible under conditions where the bee is fixed in a holder, so that environmental factors can be fully controlled and the application of stimuli occurs in a precisely defined manner (Kuwabara 1957, Bitterman *et al.* 1983, Matsumoto *et al.* 2012, Sommerlandt *et al.* 2014). Memory formation after single-trial learning can last for days, and with three-trial conditioning in free-flying bees the memory can persist for the whole life time (Hammer and Menzel 1995). Since associative learning, in particular the classical Pavlovian type, is well described on a phenomenological and operational level, it serves as an adequate method to study the neuronal and molecular processes that underlie learning and memory formation.

The cellular memory and its phases

Memory in honeybees, similar to mammals, can be divided into different phases, depending on the duration of the memory and the underlying molecular processes, which include activation of second messenger cascades, transcription and translation (**Fig 1**; Menzel 2001, Müller 2002). Repeated learning trials first induce the formation of a short-term memory (STM), which lasts for minutes to few hours. The mid-term memory (MTM) holds for up to one day, and in parallel, information can be stored as long-term memory (LTM), which lasts up to lifetime. LTM in turn can be further subdivided into an early (eLTM, up to three days) and late (lLTM, up to life time) form. On a cellular level, these memory phases are characterized by different molecular mechanisms and pathways (**Fig 2**).

Consolidation of the STM (**Fig 2**, ①) coincides with a sustained neuronal depolarization, based on a strong and persisting activity increase of the cAMP-dependent proteinkinase A (PKA) in distinct parts of the brain (Erber *et al.* 1980, Müller 1997, Müller 2000). Here, a single stimulation causes a transient release of biogenic amines, e.g. octopamine (OA; e.g. released by the VUMmx1 neuron), which leads to a modification of the respective preexisting trans-membrane receptor (AmOA1). The receptor then activates via a G protein the enzyme

adenylyl cyclase (AC; Pérez-Braun *et al.* 1994, Müller 1997, Sudlow and Gillette 1997), which converts ATP to cyclic AMP (cAMP). cAMP in turn functions as a second messenger and leads to a release of the catalytic subunits (orange ovals) of the cAMP-dependent protein kinase A (PKA) by binding to its regulatory units. The catalytic subunits of PKA can then phosphorylate components of the exocytosis machinery or K^+-channels in the presynaptic terminals. Phosphorylated and hence closed K^+ channels lead to reduced K^+ currents, which facilitate an elevated charge-dependent influx of Ca^{2+} ions with each incoming action potential. In this way, the excitation of synapses can be prolonged and is more sensitive to a repeated input of the same type of stimulation.

Repetition of neuronal stimulation activates cellular processes characteristic for the formation of a MTM (**Fig 2**, ②), which is, for example, more resistant to controversial information than STM (Menzel 1979). In general, the formation of MTM is dependent on the proteolysis of

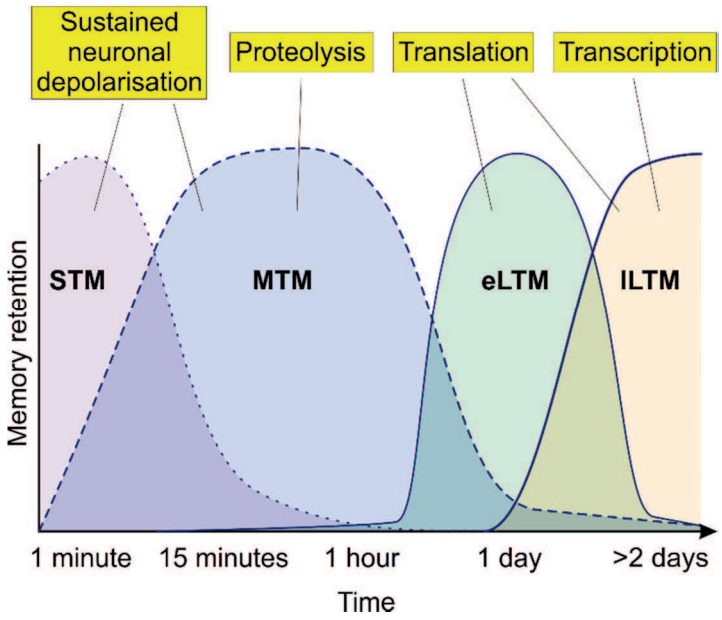

Fig 1 *Memory phases in honeybees*. Depending on the duration of the memory storage and associated cellular processes (yellow boxes), different memory phases can be distinguished. Short-term memory (STM) spans minutes up to a few hours. Mid-term memory (MTM) lasts for several hours and decays after one day. Information stored in long-term memory (LTM) can be retrieved after days. Depending on the necessity of gene transcription, LTM can be subdivided into an early (eLTM) form, lasting for up to three days, and a late (lLTM) form, where memories can be stored for life time. In contrast to the formation of an eLTM, which coincides with the translation of locally stored synaptic mRNA into protein (Puthanveettil 2013), lLTM also requires de novo gene transcription (Friedrich et al. 2004).

regulatory subunits of kinases. Prolonged elevated levels of cAMP allow the catalytic subunits of PKA to recruit the mitogen-activated protein kinase (MAPK; also called ERK), and both kinases then translocate to the nucleus (Martin *et al.* 1997). There, PKA and MAPK activate

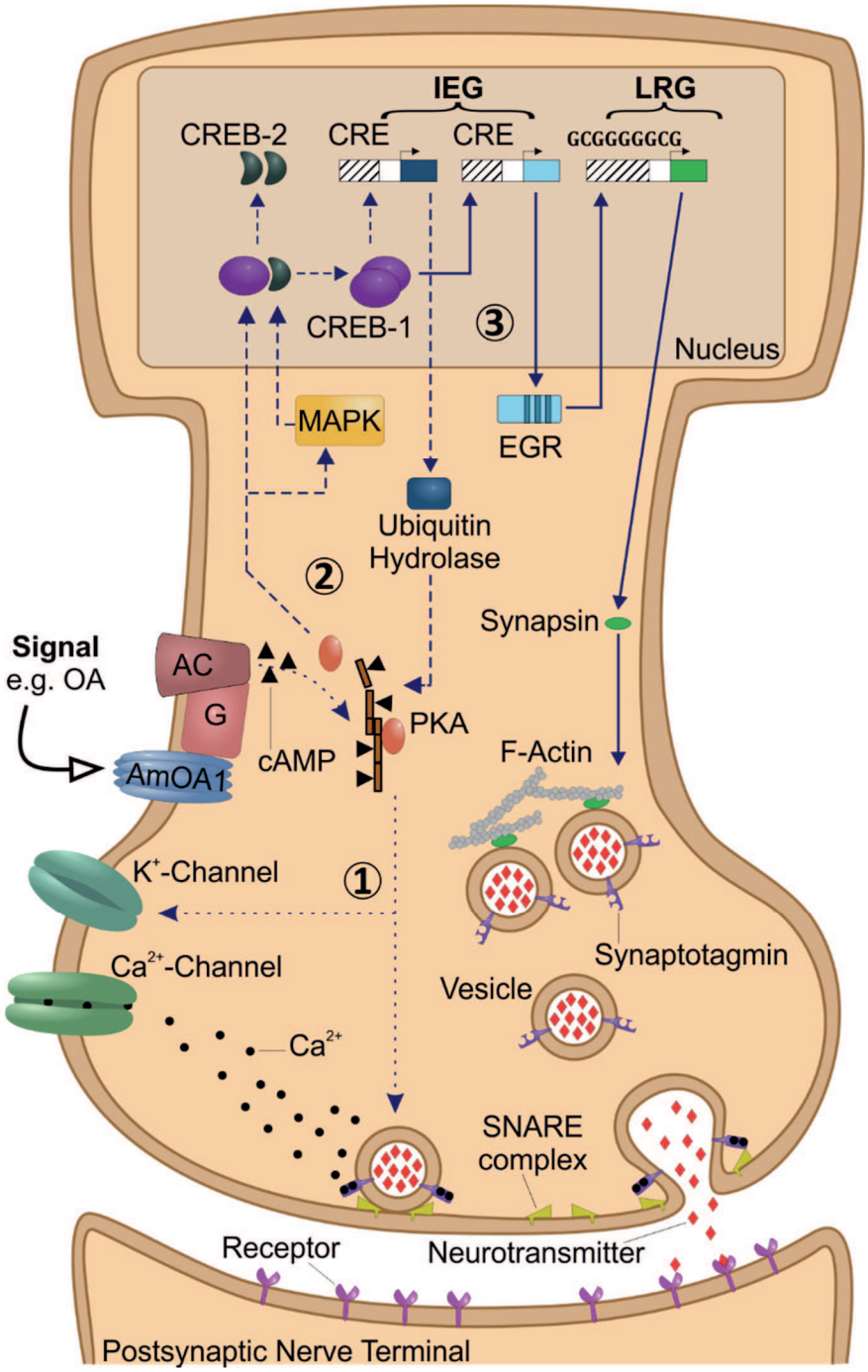

Fig 2 *Molecular pathways of memory* (concept partially adapted from Kandel 2001). Examples for molecular pathways associated with neuronal sensitization processes involved in the formation of different memory phases: short-term memory (STM; ①), mid-term memory (MTM; ②) and long-term memory (LTM; ③). See text for details.

through phosphorylations the cAMP response element-binding protein (CREB-1) by causing the dissociation from the repressive CREB-2 (reviewed in: Kandel 2012). In turn, homodimerized CREB-1 induces the transcription of immediate early genes (IEG, see below) carrying the CRE consensus sequence (5'-TGACGTCA-3' ; Montminy 1997) in their promotor region.

A central IEG activated by CREB-1 is the ubiquitin hydrolase, which is necessary for a controlled proteolysis of the regulatory subunit of PKA. Cleavage of this subunit results in a persistent phosphorylation activity of PKA and hence a further prolonged transmission at the synapse (Müller 2000; pathway discovered in *Aplysia*: Hegde *et al.* 1997, also present in humans: Jarome and Helmstetter 2013; homologs found in the honeybee genome: Gene ID 409387).

A complementary, also protease-dependent pathway underlying MTM is the Ca^{2+}-/phospholipid-induced activation of the protein kinase C (PKC) and its cleavage (by calpain) to the constitutively active protein kinase M (PKM) (Müller 2002). PKM in turn activates the Ras-MAPK (Ras: Rat sarcoma; MAPK: mitogen-activated protein kinase) pathway and hence potentially regulates gene transcription (Caruso *et al.* 2014). In both cascades, the constitutive activation of kinases (PKA and PKM, respectively) can be seen as the molecular substrate for memories.

In parallel to processes underlying the formation of the MTM, the machinery for the formation of a LTM (**Fig 2**, ③) gets activated. Memories lasting for one day and longer require functional gene transcription and the synthesis of new proteins. Regulated by constitutive transcription factors, like CREB-1, expression of target genes is induced. These target genes function as the first genomic response to neural stimulation and are referred to as immediate early genes (introduced in detail below), which can act either as direct effectors (like the ubiquitin hydrolase, which is involved in MTM), or as inducible transcription factors (like the early growth response protein, EGR; Christy *et al.* 1988, Julie Lee *et al.* 1995). The latter, in turn, induce the expression of late response genes (LRG), which are required for neuronal growth. EGR, for example, binds to its specific consensus sequence (5'-GCG GGG GCG-3') present in the regulatory region of many genes. One potential target gene is *synapsin I* (humans: Thiel *et al.* 1994), which encodes the synapsin I protein that is involved in vesicle storage and hence necessary for neoformation of synapses.

Immediate early genes in the honeybee

Long-term plastic changes in the interconnectivity of the brain neuronal network, which facilitate memory formation, depend on an early alteration of gene expression. By activating a genetic response, short-term neuronal activity can be transformed to long lasting structural changes, like rearrangements of synapses, and long-term consequences on the synaptic transmission can be initiated. The first genes which are activated in this process are under the control of constitutively expressed transcription factors and thus do not require *de novo*

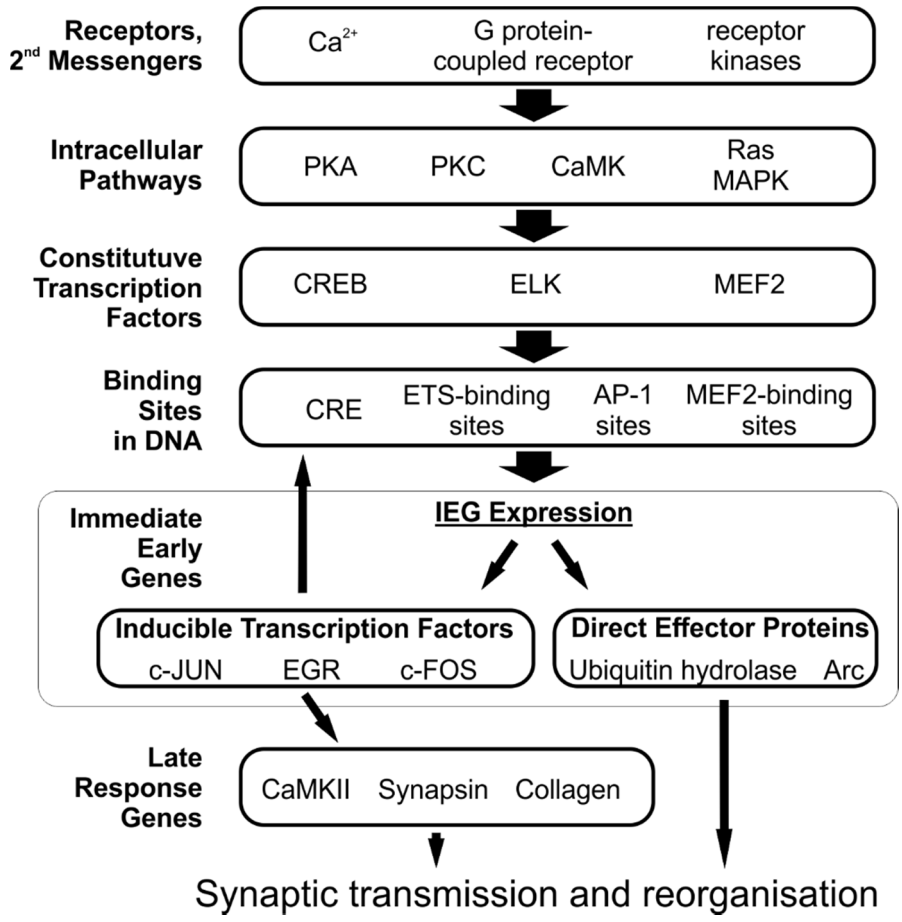

Fig 3 *Intracellular pathways underlying transcription-dependent structural plasticity in neuronal cells* (adapted and modified from O'Donovan et al. 1999, Clayton 2000). Successive stages of intracellular signal transduction lead from a stimulation of transmembrane receptors via intracellular pathways to a transcriptional activation of IEG (highlighted by grey edging), which then activate the machinery for a persistent reorganization of the neuronal network. In this context, IEG activation can be seen as the first genomic response to sensory stimulation. Within the black boxes are listed molecular representatives of each stage.

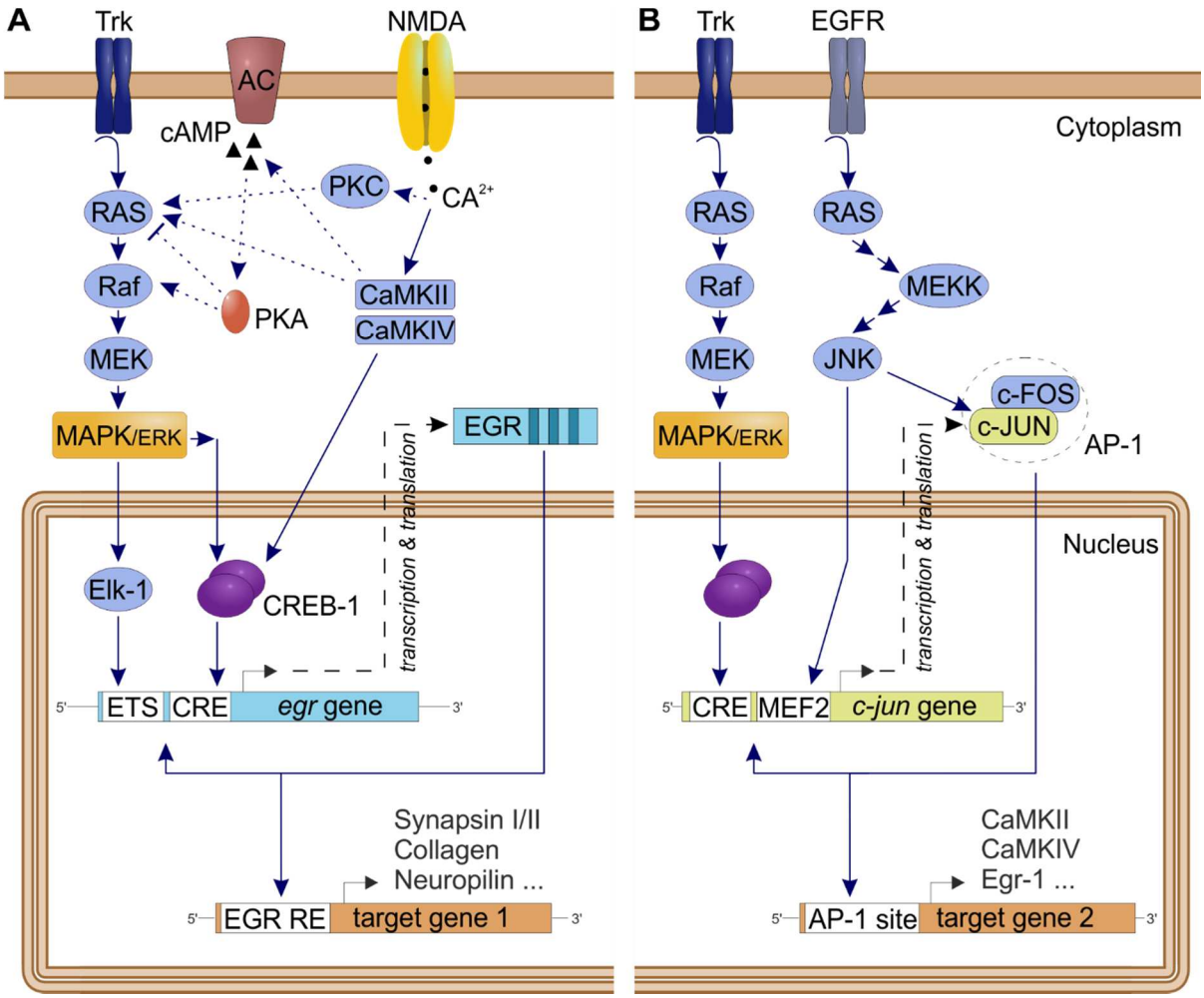

Fig 4 *Exemplified activation pathways for the immediate early genes egr and c-jun.* **A** Activation of tyrosine receptor kinases (trk) by neurotrophins induces via Ras (G protein) and Raf (kinase) the MAPK/ERK pathway, resulting in an activation of the transcription factors (TF) Elk-1 and/or CREB-1. By binding their consensus target sequences (ETS and CRE sites), the TFs induce the transcription of *egr*. The EGR protein product in turn functions as a TF and activates the transcription of various late response target genes. EGR additionally auto-regulates its own expression by interacting with the promotor of the *egr* gene. Alternative regulation pathways include the cAMP-PKA signaling pathway and NMDA receptor-mediated activation of PKC or CaM kinases. **B** Activation of *c-jun* is also mediated by the MAP/ERK pathway. Another MAPK signaling pathway includes the c-jun NH_2-terminal kinase (JNK), which activates *c-jun* expression by binding of the MEF2 site in the promotor. c-JUN protein is regulated through phosphorylation by JNK and forms homo- or heterodimers (e.g. with c-FOS) resulting in the activator protein 1 (AP-1) complex, which regulates gene transcription via AP-1 binding sites on the DNA. c-JUN also auto-regulates its own transcription. Thiel et al. (1994), Clarke et al. (1998), Davis et al. (2003), Knapska and Kaczmarek (2004), Lopez-Bergami et al. (2007)

protein synthesis. Since their transient induction occurs within minutes or hours, they are called immediate early genes (IEG). The group of IEG comprises various genes, whose protein products serve diverse cellular functions and can either act as direct effectors or as inducible transcription factors regulating downstream late-response genes (**Fig 2** and **Fig 3**; reviewed in Tischmeyer and Grimm 1999, Clayton 2000). In the nervous system, the genes regulated by IEG mainly have implications for long-lasting changes in inter-synaptic signal transduction and neuronal reorganization. The particular lure of investigating IEG lies in their potential use as mapping tools for neuronal activation due to their rapid and transient expression in response to cellular stimulation, e.g. depolarization. Various IEG were first discovered in vertebrates and are associated with complex behaviors like song-learning in songbirds (Mello and Ribeiro 1998) or experience-dependent spatial orientation in rats and mice (Guzowski *et al.* 2001, Mataga *et al.* 2001). The common conclusion of these studies regarding the properties of a stimulus that elicits an induction of IEG expression revealed two main requirements: the stimulus must firstly be biologically relevant and secondly of a certain novelty for the recipient (Mello *et al.* 1992, Papa *et al.* 1993, reviewed in Clayton 2000). A repeated presentation of a relevant stimulus that elicits an IEG induction leads to a decrease of the response strength. Typically, the expression of an IEG mRNA starts only minutes after presentation of a stimulus, with a peak transcription level within 30-60 minutes after stimulation. Afterwards, the mRNA level rapidly declines to baseline level. At the protein level, the response occurs with a certain time delay. Maximum protein levels are reached around one to three hours post-stimulation and then decline over hours, or even days (Müller *et al.* 1984, Sheng and Greenberg 1990, Morgan and Curran 1991, Zangenehpour and Chaudhuri 2002). Numerous IEG, particularly those encoding inducible transcription factors, are highly conserved among vertebrates (Long and Salbaum 1998, Burmeister and Fernald 2005), and conserved orthologs can also be found in insects (Fujita *et al.* 2013, Ugajin *et al.* 2013). Nevertheless, knowledge about the functional role of IEG in insects is scarce, and whether these genes can be used as mapping tools for neuronal activity, as in vertebrates, is an open question. In honeybees, so far only a few studies utilized IEG expression to study neuronal activation and focused on only few candidates: *C-fos* (Fonta *et al.* 1995), *c-jun* (also known as jun-related antigen, *jra*; Alaux and Robinson 2007, McNeill and Robinson 2015) and *egr* (also known as zenk, zif/268, NGFI-A; Lutz and Robinson 2013, Ugajin *et al.* 2013), which are orthologs of known and established vertebrate IEG; as well as *kakusei* (Kiya *et al.* 2007, Kiya *et al.* 2008), a non-coding RNA with unknown function.

Since *egr,* encoding a zinc finger-type transcription factor, and *c-jun,* encoding a basic leucine-zipper protein, belong to the best studied IEG in vertebrates with deep understanding of involved signal pathways (**Fig 4**), I will focus on these promising genes in the present thesis (*Chapter III* and *IV*).

Neuroanatomy and the visual pathway in social bees

A central role in processes of learning and memory in honeybees and other insects play the mushroom bodies (MB; **Fig 5**; Heisenberg 1998, Strausfeld *et al.* 1998, Heisenberg 2003, Fahrbach 2006). These paired brain centers comprise functional properties comparable to the hippocampus in mammals and undergo experience-dependent changes in volume and interconnectivity of their neuronal network (Mobbs 1982, Farris *et al.* 2001, Menzel and Giurfa 2001, Groh *et al.* 2006, Groh and Rössler 2011, Rössler and Groh 2012, Scholl *et al.* 2014). The honeybee MB neuropil is subdivided into the calyx (input) and pedunculus (output), with the first being further divided into lip, collar and basal ring (Mobbs 1982, Gronenberg 2001, Strausfeld 2002). Olfactory information is mainly integrated in the lip, whereas visual information is processed in the collar. The basal ring integrates information of both modalities. Synaptic contacts between input neurons (e.g. ascending from antennal lobes or optical lobes) and intrinsic MB neurons, so called Kenyon cells (KC), are organized in separate units, the microglomeruli (MG), which comprise a single axonal bouton and multiple dendritic spines (Groh *et al.* 2004, Groh *et al.* 2006, Groh *et al.* 2012). The number and density of MG is a measure for synaptic plasticity, since it e.g. increases in the lip after the formation of a specific olfactory LTM (Hourcade *et al.* 2010, Falibene *et al.* 2015). While many studies in the past focused on the mechanisms underlying olfactory memory consolidation, currently little is known about the functional role of the MB in the formation of different potential forms of visual memories. However, the visual pathway from peripheral sensory organs to the MB has been substantially elucidated in recent years (**Fig 5B**).

Main sensory organs for visual information in bees are the paired compound eyes, each comprising of approx. 5300 ommatidia in honeybee workers (Seidl and Kaiser 1981, Streinzer *et al.* 2013) and 3000-5600 ommatidia in bumblebee workers (Spaethe and Chittka 2003). In each apposition eye-type ommatidium, 9 photo-sensitive retinula cells are expressed in a circular array belonging to three types of spectral receptors: short-wavelength-sensitive (SWS, excitation peaks at λ_{max} = 344 nm), medium-wavelength-sensitive (MWS, λ_{max} = 436 nm) and

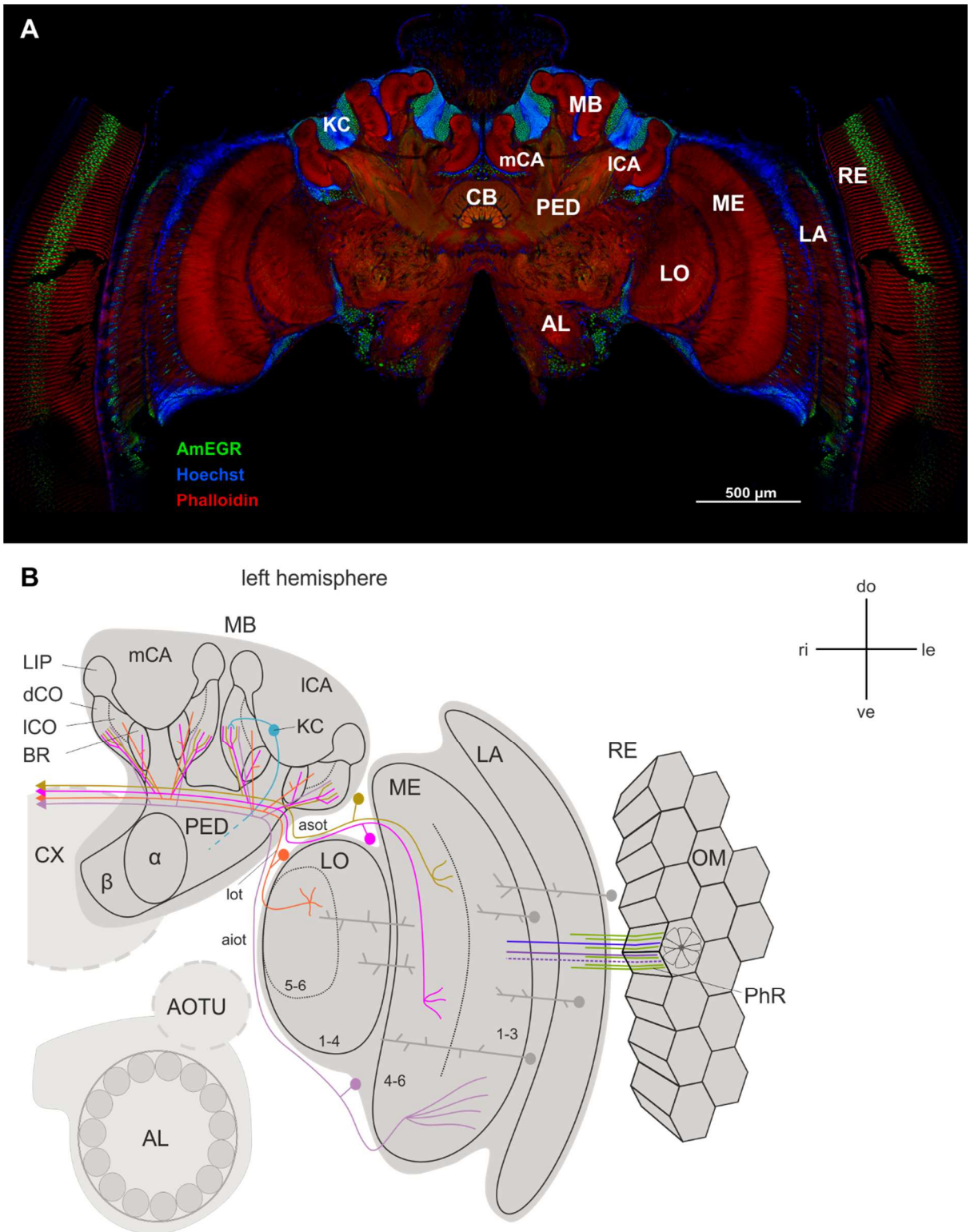

Fig 5 *Honeybee brain anatomy and visual input to the mushroom bodies (MB)*. **A** Immunofluorescence image of a frontal section through a honeybee brain, labeled for the IEG AmEGR, F-actin (through phalloidin) and cell nuclei (HOECHST). **B** Visual pathways from the compound eyes to the MB. α, alpha lobe; AL, antennal lobes; AOTU, anterior optic tubercle; ▼

▲ β, beta lobe; BR, basal ring; CA, calyx (mCA, medial; lCA, lateral); CB, central body; CO, collar (dCO, dense; lCO, loose); CX, central complex; do, dorsal; KC, Kenyon cell; LA, lamina; le, left; LIP, lip; LO, lobula; ME, medulla; OM, ommatidia; PED, peduncle; PhR, photoreceptors; RE, retina; ri, right; ve, ventral; numbers in ME and LO indicate layers; optical tracts: asot, aiot, lot. For details see text.

long-wavelength-sensitive (LWS, λ_{max} = 544 nm) (Peitsch *et al.* 1992). With respect to the photoreceptor-type composition, three ommatidia types can be distinguished: all comprise 6 LWS receptors, and type I ommatidia (44% of ommatidia) additionally contain one SWS and one MWS receptor. Type II ommatidia (46%) contain two SMS receptors, and type III ommatidia (10%) additionally contain two MWS receptors (Spaethe and Briscoe 2005, Wakakuwa *et al.* 2005). The sensitivity of the basal ninth receptor in all ommatidia types remains unclear. The axons of the photoreceptor cells project to the lamina (LWS) and outer medulla (SWS, MWS), respectively (Ribi 1975), where the visual information gets dissected (Paulk *et al.* 2009b, Dyer *et al.* 2011). While the lamina principally processes achromatic information from the LWS receptors, the medulla receives input from SWS and MWS receptors, as well as from lamina monopolar neurons. Within the medulla, broad-band, narrow-band and color-opponent neurons further separate the visual information, and projections innervate lobula outer and inner layers, with the former being motion-sensitive and largely achromatic (broad-band and narrow-band neurons), and the latter being color-sensitive (color-opponent neurons; Paulk *et al.* 2008, Paulk *et al.* 2009a; information-segregating interneurons are represented by grey branchings in **Fig 5B**). From the medulla, two tracts directly project towards the lateral and medial MB calyces (Ehmer and Gronenberg 2002, Sinakevitch *et al.* 2011): the anterior superior optic tract (asot; magenta and ocher in **Fig 5B**), which carries information from the dorsal medulla, and the anterior inferior optic tract (aiot*;* purple in **Fig 5B**), collecting excitations from the ventral part of the medulla. A third tract, the lobula tract (lot; orange in **Fig 5B**), innervates the calyces ascending from the inner lobula layers (Paulk and Gronenberg 2008). All mentioned optic tracts also project to the contralateral mushroom body, innervating the calyces of the opposite hemisphere (Ehmer and Gronenberg 2002). Within the calyces, visual information is strictly segregated (Gronenberg 2001, Paulk and Gronenberg 2008): the lot from the lobula exclusively terminates in the loose collar and the basal ring, whereas projections from the medulla separate into five layers in the

dense collar and partially innervate the basal ring (solely via the asot). As mentioned above, the projecting neurons then connect with MB intrinsic neurons, the Kenyon cells, and form synaptic complexes, called microglomeruli (Groh *et al.* 2004, Groh *et al.* 2006). As different types of Kenyon cells branch into distinct parts of the MB, thereby forming the calyces with their dendrites and the pedunculus and lobes with their axons, sensory information of different modalities (e.g. visual, olfactory, gustatory) gets integrated and associations, the basis of learning and memory, can be formed (Strausfeld 2002, Farris 2005, Menzel 2014).

Besides the projections to the MB calyces, medulla and lobula neurons (predominantly from the outer layers) additionally project to the posterior protocerebrum, like the thoracic ganglion, to connect the visual system with motor centers (Gronenberg and Strausfeld 1990). Moreover, neurons from the inner medulla and lobula layers also innervate the anterior lateral protocerebrum, like the anterior optic tubercle (Mota *et al.* 2011), through which the visual information is passed to central parts, like the central complex (Barth and Heisenberg 1997). These pathways presumably enable the integration of visual information from the compound eyes with ocellar input (Milde 1988) and motor output for the control of behaviors related to orientation (e.g. sky compas orientation and polarized light vision; reviewed in Pfeiffer and Homberg 2014)

Even though there are differences in behavioral performances (Dyer *et al.* 2008, Morawetz and Spaethe 2012), the neuronal architecture and most likely the entire visual pathway is highly conserved among the closely related honeybees (*Apis spec.*) and bumblebees (*Bombus spec.*), even on the level of individual neurons (Roig-Alsina and Michener 1993, Mares *et al.* 2005, Paulk and Gronenberg 2008, Groh and Rössler 2011). This allows for an execution of experiments with choosing the study organism according to technical requirements.

Significance of this dissertation research

Although honeybees and bumblebees have only a fraction of the number of neurons compared to mammals, they are capable of an astonishing variety of visually-guided behaviors and can master complex learning and memory tasks. By unraveling the mechanisms underlying the formation of visual memories in bees, we may begin to understand the neuronal circuitry that facilitates the long-term storage of information in the brain. Therefore, I wanted to address the following issues in the present dissertation:

First, I aimed to determine whether the formation of a visual LTM is, in analogy to olfactory LTM, dependent on gene transcription. As elaborated in *chapter II*, I trained individual bumblebees to monochromatic light stimuli in a proboscis-extension response (PER) assay (method adapted from Lichtenstein *et al.* 2015). Following a three hour resting phase, the bees were divided into three groups: the first group received a thoracic injection of the transcription inhibitor actinomycin D, whereas a second and third control group was either injected with phosphate buffered saline (PBS) or did not receive any injection. All bees were then tested for memory retrieval six hours (MTM) and three days (LTM) after conditioning. In this way, the necessity of gene transcription for the formation of a stable visual LTM was examined. Bumblebees were chosen as study organism due to their astonishing robustness against thoracic injections, and the relatively easy implementation of visual PER conditioning as compared to honeybees.

In the next part of my dissertation, I aimed to study the role of immediate early genes in early transcription processes. The two genes of interest were *Amjra* (*c-jun*) and *Amegr* (*egr*/*zif268*), which are well established in vertebrate research and known to be associated with learning and memory processes. In *chapter III*, I analyzed the spatial transcriptional characteristics of the two IEG in response to simple sensory input. For this purpose, individual honeybees were exposed to either light stimulation or an alarm pheromone component, and the expression levels of the two IEG were determined after 30 minutes in different brain parts and compared to an untreated control group. With this study, stimuli-response differences between the two genes, but not between the investigated brain neuropils, were shown. This work was an important first step for the functional characterization of the analyzed genes.

In *chapter IV*, I focused on the temporal gene expression pattern of *Amegr* during associative visual learning in the mushroom bodies. Individual honeybees were trained in an absolute PER conditioning paradigm to a monochromatic light stimulus. Following conditioning, the bees were divided into three groups with respect to sampling points: *Amegr* gene expression was examined after 30 minutes, 90 minutes and 240 minutes, respectively. Additionally, a control group was dissected without conditioning. Analysis was performed on an isoform-specific level and hence provided detailed insights into the temporal characteristics of *Amegr* expression. Moreover, by applying immunofluorescence imaging techniques with custom-made antibodies directed against the *Amegr* protein product, the spatial distribution of AmEGR was analyzed in the honeybee brain.

In the last part of my dissertation, I studied the consequences of fine color discrimination memories on the mushroom body neuronal connectivity. Despite much evidence for a major role of the mushroom bodies in the formation and retention of associative memories, knowledge about the underlying mechanisms, especially in visual learning, is sparse. Since formation of an olfactory LTM in honeybees is associated with changes in the synaptic architecture of the MB network, I aimed to find analog coherencies in visual learning. As described in *chapter V*, I trained free-flying honeybees individually in either absolute or differential conditioning to two perceptually similar colors and subsequently tested them in a non-rewarded discrimination test with both colors presented. Since only bees trained in differential conditioning successfully chose the previously learned color, differences in the impact on the neuronal network of the mushroom bodies were proposed among both groups. To clarify this issue, all tested bees were kept individually for three days in darkness to allow LTM to be formed, before the brains were dissected and analyzed for microglomeruli number and density in the lip and collar of the calyx. In combination with a correlation analysis, this study provided new insights into the coherencies of neuronal properties and behavioral performances on the level of individuals.

Chapter II – Post-conditioning suppression of gene transcription inhibits the formation of a visual long-term memory, but not mid-term memory, in the bumblebee, *Bombus terrestris*

Introduction

Bumblebees are important pollinators of angiosperm plants, due to their efficient and persevering foraging behavior. Workers collect nectar and pollen in order to supply nestmates of the colony with all required nutrients. To facilitate efficient foraging, bees must find and identify potential food sources, learn and remember how to handle the flowers to receive the nourishment, and eventually must find their way back to the nest. The main flower features that are used by bees to recognize potential food sources are olfactory and visual in nature (Odell *et al.* 1999, Chittka and Raine 2006, Kulahci *et al.* 2008), and stimuli of both modalities can be easily learned by bees in association with food reward (reviewed in Giurfa 2007). Appetitive learning initiates a sequence of transient memory phases arranged both sequentially and in parallel (*Chapter I*, **Fig 1**), finally leading to a long-lasting storage of information (Menzel 1999, Menzel 2001, Menzel and Giurfa 2001, Menzel 2012). A paired presentation of a conditioned stimulus (CS), e.g. a color or an odor, with an unconditioned stimulus (US), e.g. sucrose solution, causes associative learning and induces the formation of a short-term memory (STM; Erber 1975, Erber *et al.* 1980). The STM lasts for seconds up to minutes and is characterized on the cellular level by enhanced and prolonged neuronal depolarization facilitated by an activation of the cAMP/PKA signaling cascade and the PKC pathway (Menzel 1979, Klein and Kandel 1980, Menzel and Müller 1996, Müller 2000). Repetition of the associative presentation of CS and US facilitates memory consolidation and leads to a storage of information in the mid-term memory (MTM) that lasts for hours up to one day (Müller 2002). On the molecular level, formation of MTM includes a prolonged activation of the kinases PKC/PKM and PKA due to proteolytic processes (Müller 1997, Müller 2000, Müller 2002, Kandel 2012). Information that can be retrieved after more than one day is stored in the long-term memory (LTM; Menzel 1999, Eisenhardt 2014). Formation of a stable LTM is facilitated by a reorganization of the neuronal network, including strengthening of existing synapses, as well as synapse neoformation and degradation (Bailey and Kandel 1993, Lamprecht and LeDoux 2004, Hourcade *et al.* 2010, *Acromyrmex ambiguus*: Falibene *et al.*

2015). In contrast to STM and MTM, the processes underlying the formation of LTM requires *de novo* synthesis of proteins in both vertebrates (Hernandez and Abel 2008, Jarome and Helmstetter 2014) and insects (*Drosophila*: Tully *et al.* 1994, wasps: Collatz *et al.* 2006). This includes on the one hand translation of newly synthesized mRNA (derived from the nucleus), but on the other hand also local translation of mRNA which is stored as RNA granules in distinct subcellular domains of neuronal cells (e.g. axons, dendrites; Krichevsky and Kosik 2001, Bramham and Wells 2007, Jung *et al.* 2012). The former process (considered as the molecular substrate of late LTM [lLTM], lasting >2 days) is rather slow but long lasting by modifying the neuron's proteome, whereas the latter process (considered as being responsible for early LTM [eLTM], lasting 1-2 days) facilitates a transcription-independent dynamic and spatially restricted adaptive response to stimulation (Bramham and Wells 2007, Wang *et al.* 2009). In honeybees, studies on the participation of protein expression for the formation of long-lasting memories revealed conflicting results: Injection of translation inhibiters affects the eLTM's resistance to extinction (emetine; Marter *et al.* 2014), but not retention of olfactory (cycloheximide; Menzel *et al.* 1993, Wittstock *et al.* 1993) and visual (cycloheximide; Wittstock and Menzel 1994) memories after one to three days. In contrast, injection of a transcription inhibitor (actinomycin D) following olfactory conditioning has been shown to reduce retention of both eLTM and lLTM (Menzel *et al.* 2001, Lefer *et al.* 2012), or exclusively retention of lLTM but not eLTM (Grünbaum and Müller 1998, Wüstenberg *et al.* 1998). While studies about molecular processes underlying memory formation in bees focused on the storage of olfactory information, knowledge about subcellular requirements for the formation of visual memories is scarce. Thus, with the present study, three major questions were addressed:

i) Do bumblebees under restrained conditions learn to associate a monochromatic light stimulus with a sucrose reward? And can they retrieve the information after six hours and after three days, periods where information is generally considered to be stored in MTM and LTM, respectively?

ii) Is the consolidation of a visual memory already completed and stable for long term at the stage of MTM, or does memory performance (on population level) improve or diminish during transition to LTM?

iii) Do the two memory stages, MTM and LTM, possess differential requirements on gene transcription to store visual information?

To address these issues, harnessed bumblebees were trained in absolute visual conditioning to a monochromatic light stimulus, using the proboscis extension response (PER) assay. After conditioning, the bees received one of three treatments including an injection of the general transcription inhibitor actinomycin D (ActD), or solely phosphate buffered saline (PBS). A third group remained untreated. Six hours (MTM) or three days (LTM) after conditioning, bumblebees were tested for specific memory retrieval, as compared to a novel color. To validate the effectiveness of thorax-injected ActD, naïve bees were injected with the drug and its kinetics in the brain and cerebral hemolymph was analyzed using UPLC-MS/MS (MTM, see methods) at different time points.

Material and Methods

Preparation of bumblebees

Commercially available colonies of *Bombus terrestris* (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) were maintained in two-chambered wooden nest boxes (size of each chamber: 240x210x110 mm) at 25°C and 70% relative humidity with a 12:12 h light/dark cycle. The bees were provided with pollen and Apiinvert (Südzucker AG, Mannheim, Germany) *ad libitum*. For conditioning experiments, bumblebee workers were randomly collected from the colonies and anesthetized on ice prior to fixation in plastic tubes by means of metal pins and adhesive tape (as described in Sommerlandt *et al.* 2014). This setup allowed the bees to freely move the proboscis, the antennae, and the first pair of legs (for US perception) in order to perform PER conditioning (**Fig 6A**). Harnessed bees were fed *ad libitum* with 30% sucrose solution (w/v) and placed in a dark climate cabinet over night at 23°C and 75% RH. Before start of conditioning, the bees were tested for an intact PER by presenting a toothpick soaked with 50% sucrose solution (w/v) to the antennae, and only bees extending their proboscis were selected for the following experiments.

Conditioning of the proboscis extension response (PER)

All selected bees were randomly assigned to one of three experimental groups. The first group (ActD) was assigned for injection of actinomycin D after conditioning, whereas the two control groups (PBS, untreated) received either PBS or no injection (see below for procedure of injection). Eight bees, which were randomly chosen from the three groups, were placed on a movable sleigh in individual chambers (50x50x60 mm) and a holder for a monochromatic filter

was placed above the first bee (setup described in Lichtenstein *et al.* 2015). The color filter (Schott & Gen, Jena, Germany) possessed a transmission maximum at 435 nm and half band width of 10 nm and was illuminated by a cold light lamp (KL1500, Leica, Wetzlar, Germany) to produce the CS. The first bee was placed in position and allowed to become accustomed to the setup for 10 s, before the CS (monochromatic light) was presented for 9 s. During the last 3 s of CS presentation, the US (50% sucrose solution) was provided in parallel as reward. Following offset of CS and US, the bee rested for another 10 s before the sleigh was moved and the next bee was placed below the filter holder. Each bee received 10 conditioning trials with an inter-trial interval (ITI) of 8 min. The extension of the proboscis during the 6 s CS-only phase was scored as a conditioned response and bees that did not respond to the sucrose with a PER in more than four US presentations were excluded from analysis.

Drug injection

To investigate the requirement of post-training gene transcription for the formation of LTM, the three experimental groups were treated as follow: the ActD group and the PBS group received an injection of 1.5 µl of either 1.5 mM actinomycin D (Carl Roth, Karlsruhe, Germany) in PBS with 1% DMSO or solely PBS with 1% DMSO. In accordance with the procedures applied in olfactory studies (Grünbaum and Müller 1998, Wüstenberg *et al.* 1998, Menzel *et al.* 2001, Lefer *et al.* 2012), injection occurred 3 h after conditioning into the dorsal thorax by means of a microliter syringe (5 µl volume; Hamilton, Bonaduz, Switzerland; setup in **Fig 7A**). The third group of bees remained untreated without any injection and served as a control for possible lesion and DMSO effects.

Memory retrieval tests

To evaluate the visual memory performance, the bees of each treatment group were further classified into a subgroup tested for MTM 6 h after conditioning, and a subgroup tested for LTM 3 days later. Before memory testing, all bees received the US delivered to the antennae to evaluate vital functions. Validation of the CS-specific memory retrieval was accomplished by placing the bees in the above described conditioning apparatus and successively presenting once the CS and a novel monochromatic light stimulus (NCol; 528 nm filter; Schott & Gen, Jena, Germany) in a random order. An individual extending the proboscis during CS presentation and not during NCol presentation was counted as displaying a specific

conditioned response. In contrast, bees showing a PER during NCol presentation or no reaction to the CS were considered as not displaying a specific conditioned response.

Evaluation of actinomycin D kinetics in brain tissue and hemolymph

The kinetic properties of ActD in the brain and hemolymph of the bumblebees determine the drug's effectiveness in inhibiting gene transcription and hence are crucial for an understanding of transcriptional waves underlying memory formation. To evaluate the temporal dynamics of injected ActD, 65 bumblebee workers were randomly collected from colonies and harnessed in plastic tubes as described for conditioning experiments. After the fixed bees were fed to saturation with 30% sucrose solution (w/v), they were maintained in a dark climate cabinet (23°C, 75% RH) over night. On the following day, bees that possessed an intact PER in response to 50% sucrose (w/v) received an injection of 1.5 µl ActD (1.5 mM in PBS with 1% DMSO; procedure as described above), and afterwards were dissected at different time points (5, 15, 30, 60, 120 or 480 min after injection). For dissection, a bee's antennae were amputated and the head capsule was opened by means of a razor blade holder. All glands and trachea were carefully removed, 2-4 µl of hemolymph were collected with a microliter syringe (Hamilton, Bonaduz, Switzerland) and the entire brain was collected in a 1.5 ml reaction tube (Eppendorf, Hamburg, Germany). Determination of the wet tissue weight was followed by immediate freezing in liquid nitrogen. The quantification of ActD in brain and hemolymph samples was accomplished in cooperation with Dr. Markus Krischke from the Department of Pharmaceutical Biology of the University of Würzburg. Ultra performance liquid chromatography and tandem-mass spectrometry (UPLC-MS/MS) with multiple reaction monitoring (MRM) was applied and 7-amino-actinomycin D (Sigma Aldrich, St. Louis, Missouri, USA) served as an internal standard for quantification (method adapted from Veal *et al.* 2003). Sample preparation and collection, as well as final data analysis and interpretation was performed by Frank Sommerlandt, UPLC-MS/MS measurements for ActD quantification was performed by Dr. Markus Krischke.

Statistical analysis

Statistics for the memory acquisition curves were done on an individual's number of conditioned responses (between 0 and 9). Comparison among group performances was performed using Kruskal-Wallis test and correlation between number of conditioned

responses and body size was evaluated by Spearman correlation test. Memory retrieval (on the number of CS-specific responses) after 6 h and 72 h was compared among groups using single factor ANOVA and Tukey-HSD post hoc analysis. Normal distribution of data was tested with Shapiro-Wilk test. Within-group comparison of memory performance after 6 and 72 hours was calculated using chi² test. Dynamic changes of the amount of actinomycin D in brain tissue and hemolymph of injected bumblebees was evaluated by means of Kruskal-Wallis test and Mann-Whitney-U post hoc pairwise comparison. All tests were performed with IBM® SPSS Statistics software Version 20.

Results

Bumblebees of the three experimental groups learned the CS (monochromatic light at 435 nm wavelength) as a predictor of the US (sucrose solution; **Fig 6B**). After ten conditioning trials, about 45 to 50% of all bees exhibited a conditioned response (ActD group, 50%; PBS group, 48%; untreated control, 45%) and no significant differences were found among groups

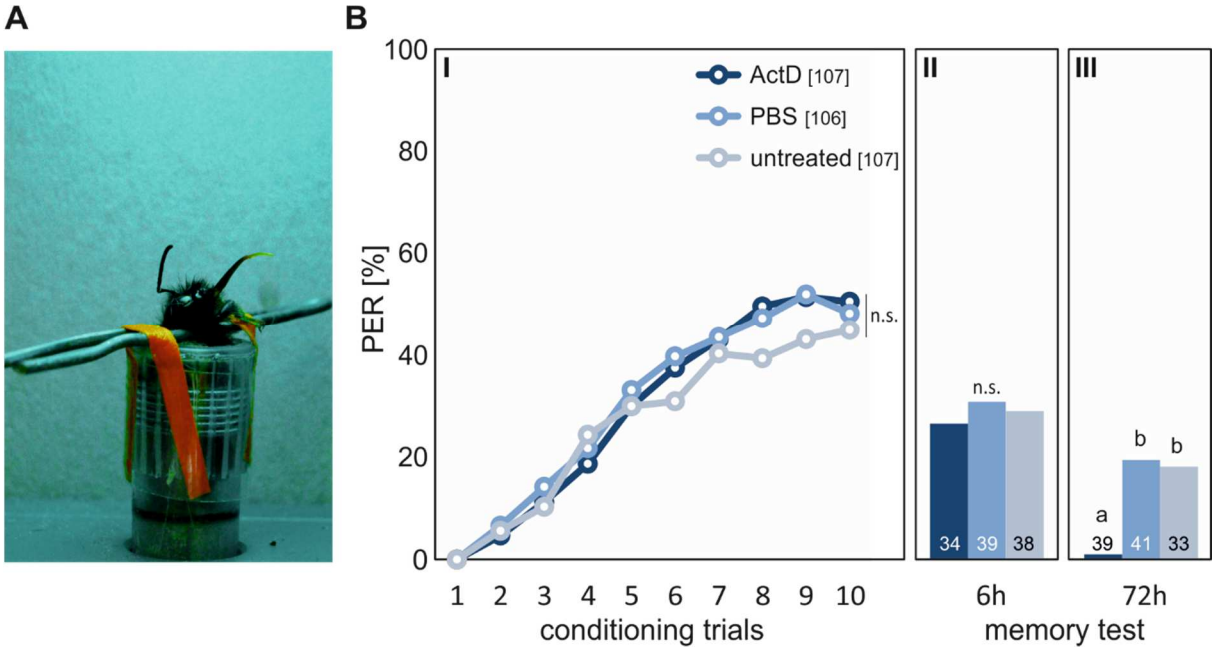

Fig 6 *Conditioning of the PER to monochromatic light and memory retrieval*. **A** A harnessed bumblebee extending the proboscis in response to monochromatic light. **B** Proportion of bees of the three experimental groups that possessed a conditioned response to CS application, plotted per conditioning trial (I) and during test for memory retrieval after 6 h (II) and 72 h (III). n.s., not significant; different letters imply significant differences. Numbers in brackets and in columns show sample size.

considering the number of conditioned PERs per individual (P=0.519, chi²=1.310). No effect of body size (measured as inter-tegulae span; ranging from 2.7 mm to 4.7 mm) on learning performance (number of PERs during CS-only presentation) was observed (P=0.916, r=0.007). When testing for the retrieval of MTM six hours after conditioning, one quarter to one third of the tested bees displayed a specific response to the previous learned CS (ActD group, 26%; PBS group, 48%; untreated control, 45%), independent of treatment group (P=0.923, F=0.080). In the final LTM test 72 hours after conditioning, 20% of the PBS-treated bees and 18% of the untreated control bees possessed a specific response towards the CS (as compared to the novel color), whereas not a single bee of the actinomycin D-treated group showed a learned response to the CS (among group comparison, P=0.014, F=4.446; Tukey-HSD post hoc analysis: ActD vs. PBS, P=0.021; ActD vs. untreated, P= 0.048; PBS vs. untreated, P=0.983). Comparison of memory performance on group level after 6 and 72 hours revealed no differences for the PBS (P=0.245, chi²=1.351) and untreated (P=0.289, chi²=1.124) groups, but for ActD-treated bees (P=0.001, chi²=11.775). To monitor the kinetics of ActD in brain tissue and hemolymph,

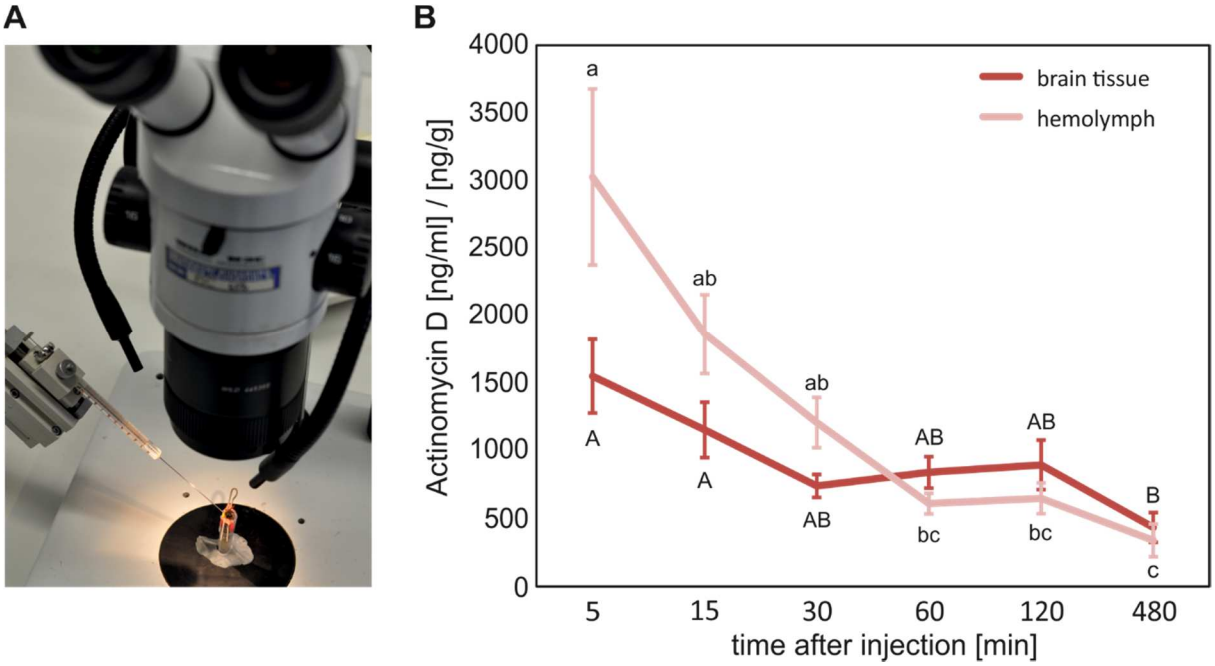

Fig 7 Injection of actinomycin D and quantitative time course analysis. **A** Injection of a harnessed bumblebee by means of a microliter syringe, monitored via binoculars. **B** Temporal course of actinomycin D quantity in brain tissue and hemolymph of bumblebees after thoracic drug injection. Given is the amount of actinomycin D per milliliter hemolymph and per gram brain tissue. Equal letters imply no statistically significant differences. N=10-11 per time point.

the levels were quantified in bees at six different time points after injection (**Fig 7B**). A significant decrease in the average amount of ActD was observed in hemolymph (5 min, 3025±650 ng/ml; 15 min, 1861±294 ng/ml; 30 min, 1206±185 ng/ml; 60 min, 610±77 ng/ml; 120 min, 643±126 ng/ml; 480 min, 339±121 ng/ml; $P<0.001$, $Z=30.052$; significance revealed by post hoc pairwise comparison: 5 min vs. 60 min, $P=0.009$, $Z=3.423$; 5 min vs. 120 min, $P=0.018$, $Z=3.242$; 5 min vs. 480 min, $P<0.001$, $Z=4.447$; 15 min vs. 480 min, $P=0.003$, $Z=3.753$), as well as in brain tissue (5 min, 1548±272 ng/ml; 15 min, 1152±203 ng/ml; 30 min, 740±84 ng/ml; 60 min, 840±116 ng/ml; 120 min, 865±199 ng/ml; 480 min, 435±110 ng/ml; $P=0.002$, $Z=19.567$; significance revealed by post hoc pairwise comparison: 5 min vs. 480 min, $P=0.001$, $Z=4.134$; 15 min vs. 480 min, $P=0.030$, $Z=3.092$) of injected bumblebees.

Discussion

With the present study it could be confirmed that bumblebees are able to associatively learn a monochromatic light stimulus as a predictor of rewarding sucrose solution in PER conditioning (**Fig 6B (I)**). In accordance with other recent studies (Riveros and Gronenberg 2012, Lichtenstein *et al.* 2015), no foregoing deprivation of the antennae (as it was suggested in earlier studies, e.g. Niggebrugge *et al.* 2009) is necessary for successful PER conditioning of visual stimuli. After ten conditioning trials, about half of the bees were able to show a conditioned response towards the color stimulus, independent of treatment group. In the untreated group, the learned information was retrievable after six hours (representing MTM; **Fig 6B (II)**) and, as here shown for the first time, also after three days, implying the formation of a stable late long-term color memory (**Fig 6B (III)**). The scarce number of studies investigating visual learning in bees applying the PER assay so far only evaluated short-term effects by analyzing STM (acquisition of memory) and MTM/eLTM (lasting for up to one day; Hori *et al.* 2006, Niggebrugge *et al.* 2009, Dobrin and Fahrbach 2012, Riveros and Gronenberg 2012, Jernigan *et al.* 2014, Lichtenstein *et al.* 2015). Long-term effects were not tested so far. It is particularly problematic to tell MTM apart from eLTM in behavioral studies, since characteristic underlying molecular processes of both memory types run partially in parallel: on the one hand, proteolysis-dependent prolonged activation of kinases, e.g. protein kinase C (PKC), is characteristic for MTM formation but can last for more than 24 hours (Grünbaum and Müller 1998, Müller 2002). On the other hand, an inducible truncated isoform of PKC, PKMζ, is stored as “dormant” mRNA at dendritic terminals and translated into protein upon induction

(Muslimov *et al.* 2004, Glanzman 2013), even though protein synthesis is considered to be characteristic for the formation of LTM (Menzel 2001, Menzel 2012). Thus, it is reasonable to clearly separate the effects on MTM from those on LTM by investigating the late phases (≥3 days) of LTM.

Consolidation of memories takes time and includes different stages of organization and reorganization: while the initial acquisition of information is a rapid process and highly susceptible to change and loss, long-term maintenance of stable memories is based on time-depending processes, including e.g. reorganization of the nervous system (Squire 1986, Stickgold and Walker 2005). It was shown for vertebrates that performance accuracy in memory retention after a learned visual discrimination task improved with time after training, even without intervening practice (Stickgold *et al.* 2000, Walker *et al.* 2003). Thus, consolidation leads to a strengthening of the memory trace. The consolidation process, based on cellular and molecular mechanisms including e.g. reorganization of the synaptic network, seems to occur in large parts during periods of rest, like sleep (Stickgold 2005, Stickgold and Walker 2005); and functional coherencies between memory formation and sleep were shown for vertebrates (birds: Brawn *et al.* 2010, rodents: Binder *et al.* 2012) as well as insects (*Drosophila*: Haynes *et al.* 2015). In honeybees, sleep has been shown to be necessary for the stable consolidation of memories about odor and navigation information (Hussaini *et al.* 2009, Klein *et al.* 2010, Beyaert *et al.* 2012). However, in the present experiments, no significant changes in memory performance during transition from MTM to LTM was observed in untreated bees on the population level (number of bees that exhibited a conditioned response). Thus, the strength of the memory of the conditioned association has been developed to the final level already on the stage of the MTM and is not improved or diminished during transition to LTM. Nevertheless, different processes are involved in the formation of MTM and LTM, indicated by different requirements of post-training gene transcription: While the ActD treated group of bumblebees was able to retrieve the learned association six hours after conditioning, bees from this group failed in the memory test after three days (**Fig 6**). These differential dependencies on post-conditioning gene transcription in the formation of MTM and LTM seems to be independent from the modality of the CS, as it was also shown for olfactory conditioning in honeybees (Wüstenberg *et al.* 1998). For associative learning of olfactory stimuli, two distinct waves of gene transcription in the course of memory formation have been suggested (Bailey *et al.* 1996, Lefer *et al.* 2012): The first wave

occurs rapidly after onset of conditioning, lasts for approx. 30-40 minutes and is considered to represent the activation of immediate early genes (IEG; Tischmeyer and Grimm 1999, Lefer *et al.* 2012; see *chapter III* and *IV*). The second wave, which is potentially caused by the IEG-dependent activation of late response genes that are involved in structural reorganization of the synaptic network, occurs presumably around three to eight hours after conditioning (Wüstenberg *et al.* 1998, Lefer *et al.* 2012). Considering this general time frame, in the present study the second transcriptional wave was effected and particularly late response effector genes were prevented from facilitating the structural plasticity required for the storage of long-lasting memories.

The so far unknown factor for determining the timing of waves of gene expression is the latency in effectiveness of the pharmaceutical drugs used to inhibit the process of transcription. The peptide antibiotic actinomycin D is derived from *Streptomyces* bacteria and impedes the RNA polymerase in its function by intercalation with the DNA (reviewed in Koba and Konopa 2005). In cooperation with Dr. Markus Krischke (Department of Pharmaceutical Biology of the University of Würzburg) the kinetics of ActD were quantified in the brain and cerebral hemolymph of thorax-injected bumblebees (**Fig 7**). A rapid decline in the amount of ActD was observed 5-30 minutes after injection in both, brain tissue and hemolymph, before the levels remained constant at approx. 650 ng/ml (hemolymph) and 850 ng/g (brain tissue) until 120 minutes after injection. The levels then further decreased after 480 min to a level of 340 ng/ml and 440 ng/g in hemolymph and brain tissue, respectively.

The translocation of ActD from thorax to head and brain occurs in a rapid fashion and therefore the time of drug injection can be considered as starting time of its intended activity. Considering the minimal effective dose of actinomycin D (ranging from 50 ng/ml effecting RNAP I and 500 ng/ml for RNAP II to 5 µg/ml with RNAP III; Perry and Kelly 1970, Bensaude 2011), the expression of class II genes (all protein coding genes) is inhibited for more than two hours after injection. This direct approach to monitor the drug's effectiveness is consistent with findings from honeybee studies, where the effective period of ActD in blocking transcription (measured by the reduction of incorporation of ^{3}H-labeled uridine into total RNA) was determined to up to 150 minutes after injection (Wüstenberg *et al.* 1998, Menzel *et al.* 2001).

In conclusion, the present study revealed three major results: First, the ability of bumblebees to associatively learn a color stimulus as a predictor of a reward in the PER assay was

confirmed and the possibility to retrieve this information after 3 days was shown. Second, the consolidation of a stable LTM has no effect on the strength of the visual memory (retrieval performance on group level) as compared to MTM. And third, the formation of LTM, but not MTM, requires functional gene transcription three to six hours after conditioning. Whether visual associative learning also induces an earlier, first transcriptional wave (as it is suggested for olfactory learning in honeybees; Lefer *et al.* 2012), needs to be further investigated and is partially addressed in *chapter IV*.

Chapter III – Impact of light and alarm pheromone on immediate early gene expression in the European honeybee, *Apis mellifera*

This chapter was largely adapted for the following manuscript: Sommerlandt FMJ, Rössler W, Spaethe J [under review]*. Impact of light and alarm pheromone on immediate early gene expression in the European honeybee, Apis mellifera.*

Introduction

In vertebrates, neuronal stimulation is associated with a rapid and transient increase in the expression of so called *immediate early genes* (IEG). The expression of IEGs is independent of *de novo* protein synthesis and reaches a maximum of mRNA level approximately 30 min after stimulation. It is therefore considered as the first genomic response of an organism to sensory input (Terleph and Tremere 2006). Moreover, IEG products are assumed to be involved in processes that facilitate neuronal synaptic plasticity (reviewed in Clayton 2000, Terleph and Tremere 2006). Based on these characteristics, IEGs are routinely used as markers for mapping neuronal activation in vertebrate brains (Guzowski *et al.* 2005). More recently there have been increased efforts to adapt this approach to insects (Kiya *et al.* 2008, Fujita *et al.* 2013). Two candidate IEGs have been proposed in the honeybee, *Amjra* (*Apis mellifera* Jun-related antigen) and *Amegr* (*Apis mellifera* early growth response protein), which encode orthologs of the widely conserved vertebrate transcription factors c-JUN and EGR, respectively (Struhl 1988, Long and Salbaum 1998, Ugajin *et al.* 2013). *Amjra* expression was shown to be induced in the antennal lobes (AL) after stimulation with isopentyl acetate (IPA; component of the alarm pheromone), and in the lateral protocerebrum, mushroom bodies (MB) and optical lobes (OL) after sucrose stimulation. *Amegr*, on the other hand, was shown to be upregulated in the MBs after orientation flights and seizures provoked by awakening from CO_2-induced anesthetization (**Table 1**). Knowledge of the functions of IEGs in honeybees is still fragmentary and most of the studies have focused on the temporal and/or spatial expression patterns of a single IEG in relation to distinct stimulus modalities or behaviors. Moreover, mRNA levels, in most cases, were measured in a single neuropil, rather than in comparison with different brain regions. Thus, here the issue is addressed of whether both IEGs show similar mRNA expression patterns in response to stimuli of different modalities (olfactory and visual), and whether the

activation of IEGs is restricted to specific neuropils in the worker honeybee brain. In addition, the first findings of an isoform-specific expression analysis of *Amegr* are reported.

Material and Methods

Two hundred honeybee workers (*Apis mellifera carnica*) that had emerged within 24 hours from a single comb with a multiple-mated queen were transferred to a wooden colony box (140x160x100mm) and maintained for seven days at 32°C in constant darkness. Apiinvert (Suedzucker, Mannheim, Germany) and pollen were provided *ad libitum*. The seven day period was chosen to match the onset of foraging, a time point when the developmental maturation of the bee brain is basically completed. Individual bees were then immobilized on ice, fixed in acrylic holders, fed *ad libitum* with 1M sucrose solution, and kept overnight in a climate cabinet (28°C; constant darkness). On the following day, individual bees were taken from the cabinet and randomly assigned to one of three experimental groups: i) The first group was subjected to light stimulation for 5 minutes applied by a cold light source (KL1500, Schott AG, Mainz, Germany). ii) The second group received olfactory stimulation by isopentyl acetate (IPA) for 5 minutes, applied through a filter paper soaked with 20 µl IPA placed at a

Gene	Stimulation	Tested/Affected Neuropil	Method	Reference
Amjra	IPA	AL/AL	qPCR	Alaux, Robinson (2007)
	sucrose	whole brain / AMMC, OL, MB, LP	ISH, qPCR	McNeill, Robinson (2015)
Amegr	Orientation flight	MB/MB	ISH, qPCR	Lutz, Robinson (2013)
	CO_2 anesthetization	MB/MB	qPCR	Ugajin *et al.* (2013)

Table 1 *Summary of studies analyzing Amjra and Amegr expression in honeybees using different types of stimulation and methods of analysis.* AL, antennal lobes; AMMC, antennal mechanosensory and motor center; IPA, isopentyl acetate; ISH, in situ hybridization; LP, lateral protocerebrum; MB, mushroom bodies; OL, optical lobes; qPCR, quantitative real-time PCR

distance of 1 cm in front of the bee. iii) Individuals of the third group served as a control and were taken from the climate cabinet for 5 minutes without treatment. All groups were treated under red light conditions. Next, 25 min after treatment, bees were decapitated and the brains were immediately dissected. Antennal lobes, optic lobes and mushroom body calyces were separated and rapidly frozen in liquid nitrogen. This schedule matched the general time course of IEG expression, which shows highest mRNA levels approx. 30 min after stimulation (Clayton 2000). RNA was then isolated according to a standard phenol-chloroform extraction protocol (see *Appendix II*). Expression levels of target mRNAs (*Amjra*, *Amegr*) were evaluated applying quantitative real-time polymerase-chain-reaction (qPCR). Therefore, total RNA of each sample was used as a template for cDNA synthesis (Reverse transcription kit; Qiagen, Venlo, The Netherlands), and qPCR reaction was performed using a Mastercycler realplex² (Eppendorf, Hamburg, Germany) in combination with a SYBR Green RT-qPCR Mix (VWR-Peqlab, Radnor, Pennsylvania, USA) and gene-specific primers (**Table 2**). Biological replicates were measured in technical triplicates. For analysis, means of C_T-values (cycle number when SYBR signal exceeds detection threshold) of target gene triplicates were calculated and, according to Vandesompele *et al.* (2002), normalized with the geometric mean of the

Target / Acc. Number	**Primer name**	**Sequence 5'-3'**	**Reference**
Amrp49 (*RpL32)* /NM_001011587.1	rp49_Fwd	CGTCATATGTTGCCAACTGGT	Lourenço *et al.* (2008)
	rp49_Rev	TTGAGCACGTTCAACAATGG	
AmGAPDH / XM_393605.5	GAPDH_Fwd	GATGCACCCATGTTTGTTTG	Scharlaken *et al.* (2008)
	GAPDH_Rev	TTTGCAGAAGGTGCATCAAC	
Amjra / XM_003250988.2	Jra_Fwd	CTGAAGGGCGAGAACAGCGAA	
	Jra_Rev	GCGACCATAGACACCATCAGACGA	
AmegrX1 / XM_001122050.3	Egr_Fwd	ACCAGCAGCGATTTACCGTCCT	
	Egr_Rev	GACTCTTGGTGATGGTGGTGTCG	

Table 2 *Reference (Amrp49 and AmGAPDH) and target (Amjra and Amegr isoform X1) genes with GenBank (NCBI, Bethesda, USA) accession numbers and corresponding primer sequences used for qPCR*

C_T-values of two commonly used housekeeping genes (*rp49* and *GAPDH*; Lourenço *et al.* 2008, Scharlaken *et al.* 2008). Normal distribution of data was tested by applying Shapiro-Wilk test and statistics with one-way ANOVA and Tukey post hoc test were performed with IBM® SPSS® Statistics software (Version 20).

Results

QPCR analysis revealed differences in the stimulation-dependent expression values of both genes. Both IPA and light exposure induced an increase in the expression of *Amjra* in all analyzed brain compartments, as compared to the control group (**Fig 8**). This effect was significant in antennal lobes (F=5.933, p=0.008; control vs. IPA: p=0.006; control vs. light: p=0.027; IPA vs. light: p=0.630), optic lobes (F=5.892, p=0.007; control vs. IPA: p=0.025; control vs. light: p=0.006; IPA vs. light: p=0.734), and mushroom bodies (F=8.186, p=0.002; control vs. IPA: p=0.039; control vs. light: p=0.003; IPA vs. light: p=0.029).
In contrast, expression of *AmegrX1* was not affected by exposure to either light or IPA in any of the analyzed brain regions (AL, F=1.613, p=0.219.; OL, F=0.436, p=0.651; MB, F=0.645, p=0.533).

Discussion

The experiments have shown that honeybees subjected to light and IPA stimulation, respectively, showed a significant increase in the expression of *Amjra* compared to the control group in all tested brain neuropils, 30 min after stimulation and independently from stimulus modality (**Fig 8**). Until now, activation of *Amjra* expression in response to alarm pheromone (and partially to plant odors) was solely measured in the AL (Alaux and Robinson 2007). The present results demonstrate that the *Amjra* expression is induced across the brain by both visual and olfactory stimuli. Thus, instead of being a specific marker for olfactory induced neuronal activation in the AL, the findings in this study suggest *Amjra* is a general indicator of activity after arousal, as it has been reported for its vertebrate ortholog *c-jun* (Papa *et al.* 1993). At the neuronal level, *Amjra* expression might be a marker for cross-modal pathways, for example, those modulating the sensitivity to sensory cues via feedback mechanisms. This may include mechanisms leading to a general sensory sensitization and enhanced attention levels. Such cross-modal modulations have been found, for example, in the noctuid moth *Spodoptera littoralis*, where predator specific noise can modify the male's sensitivity to the

female's sex-pheromone (Anton *et al.* 2011). Potential feedback neurons have been proposed, including the insect serotonin-immunoreactive (SI) neuron that branches in various brain neuropils, like the MB, central body and lateral accessory lobes, and provides feedback to the

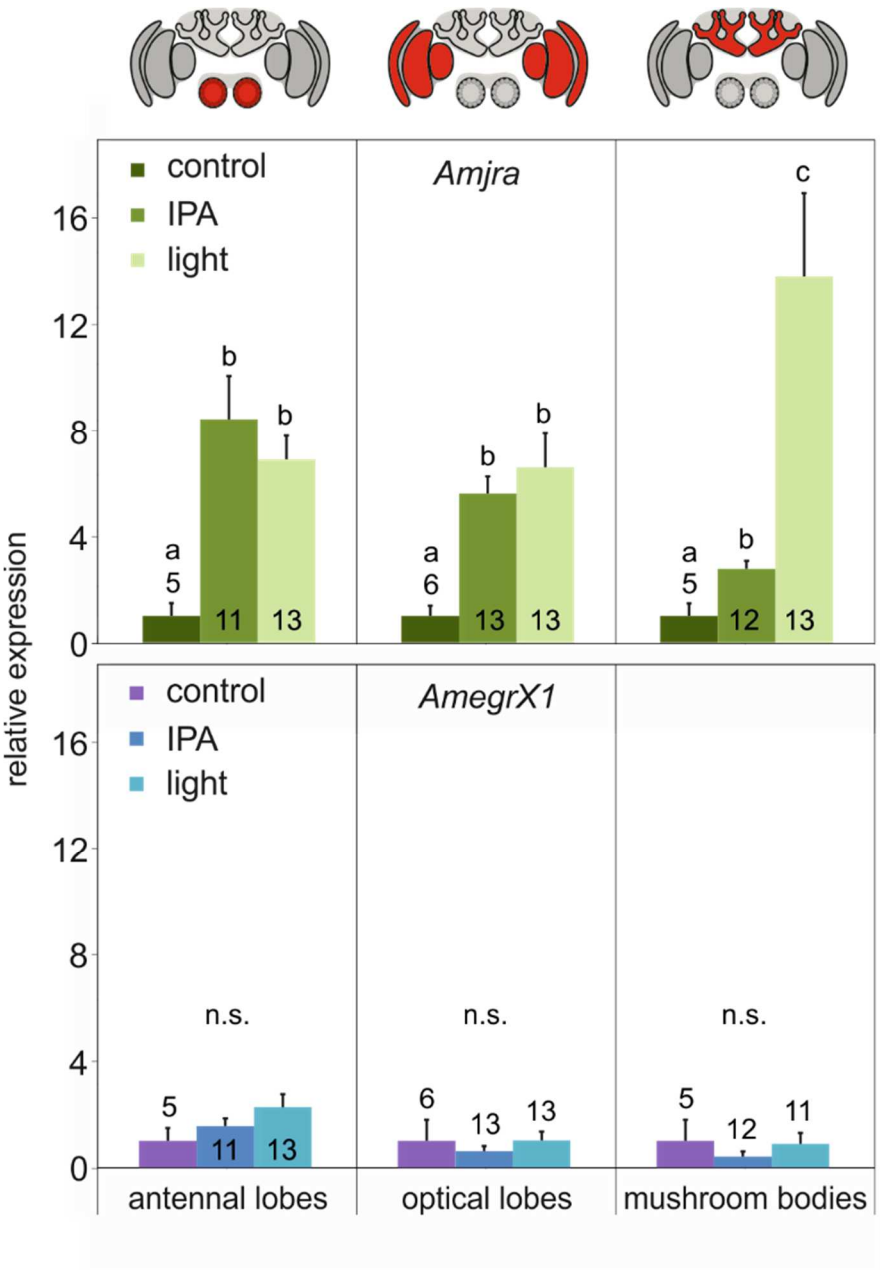

Fig 8 *Relative expression of Amjra (upper panel) and AmegrX1 (lower panel) 30 min after stimulation with IPA or light, measured in antennal lobes (AL), optical lobes (OL) and mushroom bodies (MB)*. The C_T-values were normalized against both the geometric mean of the housekeeping genes *rp49* and *GAPDH*, and then standardized to the expression levels observed in the control group. Numbers in and above bars indicate sample size; different letters indicate statistical differences. n.s., not significant

AL (Schürmann and Klemm 1984, Hill *et al.* 2002). Such a mechanism might rely on whole-brain activation by specific transcription factors like *Amjra*.

In contrast to *Amjra*, for which only one isoform is known so far, *Amegr* is expressed in at least three isoforms (**Fig 9**), which were recently identified due to an improved honeybee genome assembly with improved gene annotation (Elsik *et al.* 2014) and verified by PCR and qPCR (see *chapter IV*; **Fig 12**). We used primers partially located in the X1 isoform-specific region of exon 2 to selectively analyze *AmegrX1* (forward primer_X1_a in **Fig 12B**), which is the largest isoform and has shown to have the highest expression level in preliminary experiments. We found no significant alteration of *AmegrX1* expression after light or IPA stimulation (**Fig 8**). In contrast, previous studies in honeybees reported increased expression levels of *Amegr* following orientation flights (Lutz and Robinson 2013) and seizures (Ugajin *et al.* 2013). However, in these studies, the existence of different isoforms was not taken into account, and primers were used that amplified two (*X1* and *X2*; Lutz and Robinson 2013) or even three

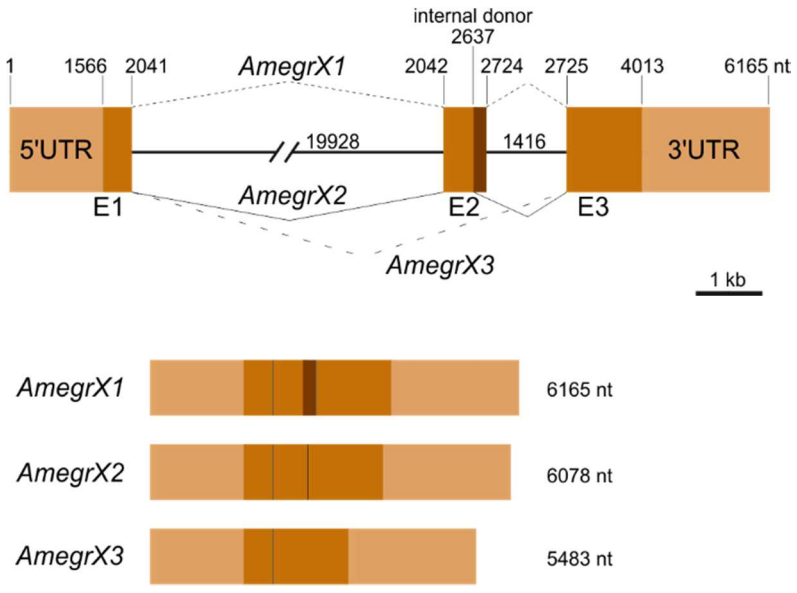

Fig 9 *Schematic representation of the three Amegr isoforms, which derive from alternative splicing*. The *Amegr* gene comprises three exons (E1-E3), which are all included in the *X1* isoform. *AmegrX2* consists of the complete exons 1 and 3, but only partially exon 2, which has an internal donor site. *AmegrX3* completely lacks exon 2. The CRE consensus sequence (5'-GACGTCA-3') which is required for activation by CREB, is located in the 5'UTR. Numbers atop of the exons indicate nucleotide numbers; solid line represents introns with corresponding number of nucleotides. Data obtained from NCBI, gene ID: 726302 (LOC726302)

isoforms (*X1-X3*; Ugajin *et al.* 2013) simultaneously. Therefore, it is not possible to determine whether the measured expression patterns relate to a particular *Amegr* isoform or whether they represent a combination of differentially expressed isoforms in various neuropils. In vertebrates, the family of EGR transcription factors is encoded by different genes and isoforms (**Fig 10**), all involved in cellular processes associated with neuronal plasticity, but activated by different stimuli and molecular signals (Beckmann and Wilce 1997, O'Donovan *et al.* 1999). Thus, it is important for future studies to identify possible isoform-specific *Amegr* expression patterns and functions by targeting single isoforms.

In conclusion, the present results suggest that *Amjra* expression in the honeybee brain is induced in response to modality-unspecific novel sensory stimulation. In contrast, *AmegrX1* is not affected by these stimuli and might instead be involved in processes underlying associative learning and memory formation, as it has been shown for the vertebrate orthologs (Jones *et al.* 2001). This and the functions of the different *Amegr* isoforms need to be further tested and is addressed in the following chapter.

Chapter IV – The immediate early gene *Amegr*: learning-dependent differential mRNA expression and protein distribution in the honeybee brain

Introduction

The early growth response (EGR) proteins are a group of immediate early gene-encoded inducible transcription factors that are involved in orchestrating differential gene expression underlying neuronal plasticity. They were first discovered in a screening assay in quest of genes implicated in the differentiation of embryonic neuroblastic rat cells into neuron-like cells, where treatment of neuroblastic cells with nerve growth factor (NGF) led to a rapid and robust increase in *egr* mRNA levels (Milbrandt 1987). Besides its activation by the neuropeptide NGF, EGR expression in cell cultures and brain tissues has been shown to be induced by a variety of pharmacological and physiological stimulations, including glutamate and NMDA, dopamine and cocaine, caffeine, ethanol withdrawal, visual and tactile stimulation, restrainment, and learning (reviewed in Beckmann and Wilce 1997). Particularly the putative role of EGR in learning and memory formation is of increasing interest in vertebrate neuroscience. Cerebral expression of EGR family members has been shown to be induced by various learning tasks including visual associative learning (macaques: Okuno and Miyashita 1996), spatial learning (mice: Fordyce *et al.* 1994, rats: Guzowski *et al.* 2001), vocal communication and auditory memory formation (zebra finches: Mello and Ribeiro 1998), as well as the formation of olfactory long-term memories (mice: Jones *et al.* 2001). In all studies, the formation of new associations was required for the activation of the *egr* genes, as sensory perception and motor responses alone did not lead to increased expression levels. Instead, members of the EGR family were shown to play a critical role in processes of long-term potentiation (LTP), which denotes a persistent strengthening of synapses that results in a prolonged synaptic transmission. More precisely, the maintenance of late phases of LTP that include gene transcription and protein synthesis and possess a prerequisite for LTM formation, requires the activation of EGR genes (Cole *et al.* 1989, Jones *et al.* 2001, reviewed in Davis *et al.* 2003).

In vertebrates, the family of EGR proteins comprises four members (EGR1 to EGR4; **Fig 10A**) that are expressed in various isoforms (Beckmann and Wilce 1997). A common structural

feature of all EGR members is a highly conserved DNA-binding domain comprising three zinc-finger motifs that target a sequence of nine consecutive nucleotides (**Fig 10B**; Beckmann and Wilce 1997, O'Donovan *et al.* 1999, Davis *et al.* 2003). Since all members target the same DNA

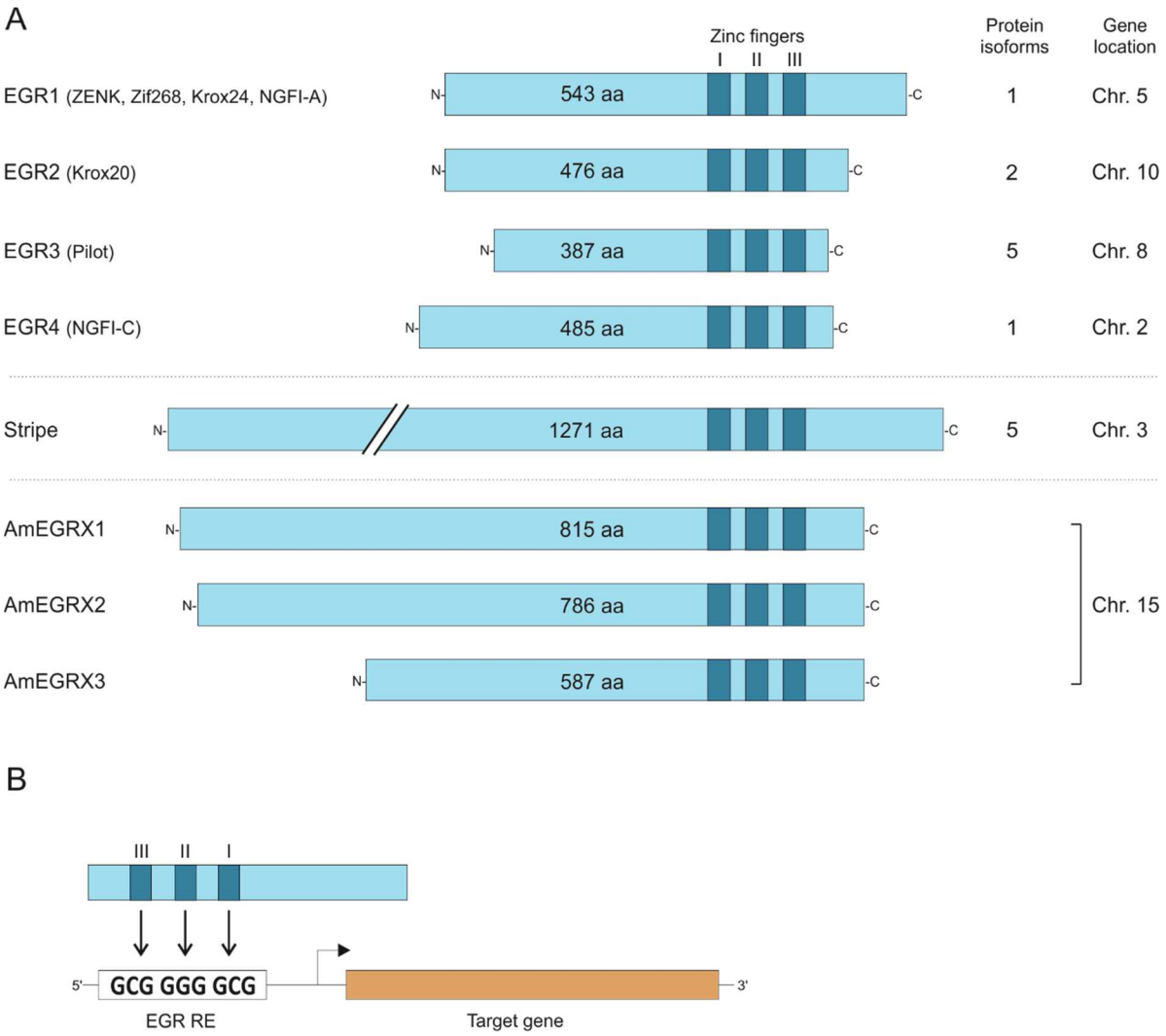

Fig 10 *Structure of EGR proteins.* **A** The vertebrate family of early growth response (EGR) transcription factors and homolog proteins in Drosophila (Stripe) and honeybee (AmEGR). Schematic alignment of the four vertebrate EGR family members (EGR1-EGR4, alternative names are listed in parentheses) with the three characteristic zinc finger motifs. As an example, the human homologs are displayed and the number of amino acids for the longest isoform, the number of known isoforms, and the chromosomal location of the genes are given (adapted from O'Donovan et al. 1999, Davis et al. 2003). Below the second dotted line, the three isoforms of the honeybee's AmEGR are shown (data obtained from the NCBI BioSystems database). The three zinc finger DNA binding domains are highly conserved. **B** Binding of EGR proteins to the canonical EGR response element (RE) upstream of potential target genes. Each zinc finger binds to three nucleotides of the RE. aa, amino acids; Chr., chromosome.

consensus sequence, differential regulation and activation of EGR proteins is facilitated by interactions of the variable peptide sequences outside the DNA-binding domain.

In honeybees, knowledge about the function and significance of EGR transcription factors is scarce. So far, only a single *egr* gene is known, located on the bee's chromosome 15 and expressed in three distinct isoforms (**Fig 9**; *AmegrX1-X3*). The transcripts vary in length from 6165 nt (*AmegrX1*) and 6078 nt (*AmegrX2*) to 5483 nt (*AmegrX3*), resulting in putative proteins containing 815 (~90 kDa), 786 (~87 kDa) and 587 (~65 kDa) amino acids, respectively. As is shown in **Table 1**, induction of *Amegr* mRNA expression was observed after orientation flights in young foragers (Lutz and Robinson 2013) and after awakening from CO_2 anesthetization (Ugajin *et al.* 2013). However, so far it is unknown whether *Amegr* shows learning-dependent patterns of differential expression and which potential variations among the isoforms might exist. Additionally, the spatial distribution of the AmEGR proteins in the honeybee brain has not been investigated so far. To address these questions, in a first step the cerebral protein distribution was analyzed using Western blotting and immunofluorescence imaging utilizing custom-made anti-AmEGR antibodies. Secondly, the temporal expression pattern of isoform-specific *Amegr* messenger RNA following visual associative learning was analyzed.

Material and Methods

Maintenance of honeybees

Approximately three hundred European honeybees (*Apis mellifera carnica*), hatched within 24 hours from a single comb, were collected in a wooden colony box (length x width x hight: 140mm x 160mm x 100mm), equipped with a wax panel and an *ad libitum* food source providing a mixture of grounded pollen and Apiinvert (Suedzucker, Mannheim, Germany). The bees were reared at 34.5°C and constant darkness for two days in a climate cabinet, followed by a reduction of the temperature to 28°C and the introduction of a 12 hour light/dark cycle. After another five to seven days, when the bees were 7-9 days of age, individuals were randomly selected for visual associative learning experiments with subsequent *Amegr* mRNA expression analysis. A same aged group of bees was dissected for examination of spatial distribution of cerebral AmEGR protein by immunofluorescence imaging and Western blot analysis.

Visual PER conditioning

Individual bees were harnessed in metal holders, fed *ad libitum* with 1 M sucrose solution and placed back in the climate cabinet. On the following day, bees were pre-tested for an intact extension of the proboscis by carefully touching the antennae with a toothpick soaked with 1 M sucrose solution. When exhibiting an intact proboscis extension response (PER), the bees were placed in the conditioning setup (as described in Lichtenstein *et al.* 2015), consisting of a movable sleigh with nine separated chambers, each providing space for a single bee, and a filter holder that housed a blue-light filter through which light from a cold light lamp shined through. The filter was a monochromatic filter (Schott & Gen, Jena, Germany) with transmission at 435 nm and a half band width of approx. 10 nm. Conditioning started when the first bee was placed below the filter holder. For accustomization, the bee could rest for 10 s until the light stimulus (conditioned stimulus, CS) was switched on and presented for 13 s. For the last 3 s (10 s after light onset), the bee was rewarded with 1 M sucrose solution (unconditioned stimulus, US) applied via a toothpick. After end of light and US presentation, the bee was given another 10 s resting phase before the sleigh was moved to place the next bee in the conditioning position. Each bee received five conditioning trials with an inter-trial interval (ITI) of 7 min and the bee's reaction was counted as a learned response when the

Target / Acc. Number	**Primer**	**Sequence 5'-3'**	**Amplicon [bp]**
AmegrX1 / XM_001122050.3	Fwd_X1_a	ACCAGCAGCGATTTACCGTCCT	170
	Fwd_X1_b	CCACCTCCTATTTCAGGCAGC	122
AmegrX2 / XM_006563423.1	Fwd_X2	CGATAAGGATAAAACAGGCAGC	123
AmegrX3 / XM_006563424.1	Fwd_X3	CACCACCCTCAGGCAGC	118
AmegrX1-3	Egr_Rev	GACTCTTGGTGATGGTGGTGTCG	

Table 3 *Primer sequences used for isoform-specific qPCR analysis*. Nucleotide sequences for forward (upper part) and reverse (lower part) primers targeting the *Amegr* isoforms. The different forward primers were combined with the same reverse primer, resulting in various lengths of PCR products (amplicon). The forward primer X1_a was used for experiments described in *Chapter III*, whereas X1_b was used in experiments described in this chapter. NCBI accession numbers are listed for the three isoforms

proboscis was extended during the 10 s CS-only phase prior to the reward presentation. The total training length required 28 min, thereby matching the general expected time course of IEG mRNA expression, which peaks approx. 30 min after (first) stimulation (first CS-US association). Only bees that exhibited a learned response in two or more trials were selected for further analysis. As a control, a group of randomly selected bees received an unpaired presentation of the stimuli: light (for 13 s) and sucrose (for 3 s), respectively, were presented alone in five trials each in a randomized order with an ITI of 3.5 min. With this procedure the total duration of the training was identical for both the paired and unpaired conditioning groups. Conditioning was performed by Leonie Lichtenstein. As a second control group, naïve bees, which received no color conditioning and just remained in their plastic holders placed in the conditioning setup without stimulation for 30 min, were sampled. After treatment, individuals were either immediately dissected, or placed back in the climate cabinet for another 60 and 180 min (90 and 240 min after first CS-US association, respectively), before preparation. As a result, three different treatment groups (paired, unpaired, and naïve) were dissected each at three different time points (30, 90, and 240 min after first association/start of conditioning). Moreover, an additional group of naïve bees was dissected right before start of conditioning experiments (time point "0"), resulting in a total of 10 experimental groups. For preparation, each bee's head capsule was opened by means of a razor blade, the brain was dissected and the calyces were collected in 2 ml Eppendorf® microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) and immediately frozen in liquid nitrogen to protect RNA from degradation.

mRNA extraction, cDNA synthesis and qPCR

The RNA of calyx tissues obtained from tested bees and respective controls was isolated as described in *Chapter III* according to a standard phenol-chloroform extraction protocol (see *Appendix II*). Expression levels of target mRNAs (*AmegrX1-X3*) were evaluated by means of qPCR. For this purpose, cDNA was obtained from total RNA (Reverse transcription kit; Qiagen, Venlo, The Netherlands), and qPCR reaction was performed using a Mastercycler (Eppendorf, Hamburg, Germany) in combination with a SYBR Green RT-qPCR Mix (VWR-Peqlab, Radnor, Pennsylvania, USA) and isoform-specific primers (**Table 3** and **Fig 12**; methods for primer validation by PCR and gel electrophoresis, see *Appendix II*). Biological replicates were measured in technical triplicates. For analysis, means of C_T-values of target gene triplicates

were calculated and normalized with the mean C_T-values of the housekeeping gene *Amrp49* (see **Table 2**).

Western blot analysis

For Western blot analysis, honeybees were anesthesized on ice and fixed in acrylic holders. The head capsule was opened and glands and trachea were carefully removed. The brain was immediately dissected by means of micro-dissecting scissors, and antennal lobes, optical lobes and mushroom bodies (main focus on calyces) were separated. 30 µl Laemmli buffer were added and the samples were immediately frozen in liquid nitrogen and stored at -70°C. Tissues were then homogenized by means of a steel ball in combination with a TissueLyser LT (Qiagen, Venlo, The Netherlands) for 3 min at 35 Hz, and denaturation of proteins was achieved by an incubation at 95°C for 5 min. Neuropils of four individuals were pooled and loaded on a 10% acrylamide gel for electrophoretic separation, followed by a Western blot protein transfer onto a PVDF membrane. After a brief rinse of the membrane with TBST, unspecific binding sites were blocked by incubation in blocking buffer for 1h at room temperature. Subsequently, the primary antibodies directed against AmEGR (immunoGlobe, Himmelstadt, Germany; originated from rabbit; 1µg/ml in TBST with 5% BSA) were applied on the membrane and incubation occurred over night at 4°C. The anti-AmEGR antibodies were directed against a peptide sequence present in all three isoforms (**Fig 11**). On the following day, the membrane was rinsed in TBST (3x5 min) and incubated with the fluorochrome-associated secondary antibodies (IRDye 680LT Donkey anti-rabbit, Li-COR, Lincoln, NE, USA ; dilution of 1:20,000 in TBST with 5% BSA) for 1h at room temperature in the dark. Following a final washing step (3x5 min TBST), the membrane was scanned using the Odyssey® Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Immunohistofluorescence analysis

For analysis of the spatial distribution of AmEGR in the honeybee brain, immunofluorescence imaging on histological brain slices was performed. For this purpose, honeybees were anesthesized on ice and fixed in acrylic holders. The head capsule was opened and glands and trachea were carefully removed. The opened head capsule of bees was immediately fixed in ice-cold 4% formaldehyde in PBS for 24 hours at 4°C. After washing in PBS (3x10 min), the brains were dissected in PBS and subsequently embedded in 5% LMP-agarose and sectioned

```
CDS       1 ATGCTGGCACTGGCG ATGCGCCGCGAACAC ACGACCGGGCGCAGC AATTACGAGCCCGCG AATCCGCTCGCGGGT
EGRX1-3   1  M  L  A  L  A   M  R  R  E  H   T  T  G  R  S   N  Y  E  P  A   N  P  L  A  G
CDS      76 TTCGACCACTTGCAG CACAACAGCGGGAAC CCGTTCGCCGTTGTG CCCTCGTCGTCGGAG GGCAGGCTTCGAGAA
EGRX1-3  26  F  D  H  L  Q   H  N  S  G  N   P  F  A  V  V   P  S  S  S  E   G  R  L  R  E
CDS     151 ACCGGCCCTGAGCCC GACGGCGAGTTGCAG GAATTCGTGGACATC GCTCAGGTACAGCAG CTTCTGCAGCAGCAG
EGRX1-3  51  T  G  P  E  P   D  G  E  L  Q   E  F  V  D  I   A  Q  V  Q  Q   L  L  Q  Q  Q
CDS     226 CAGCAGCACCAGCAG CATCAGCACCAGCAG CACCAGCAACAACAG CAGCAGCAACAACAA CAGCAGCATCAGCAG
EGRX1-3  76  Q  Q  H  Q  Q   H  Q  H  Q  Q   H  Q  Q  Q  Q   Q  Q  Q  Q  Q   Q  Q  H  Q  Q
CDS     301 CAGCAACACCAGCAG CAACAACAACAACAG CATCACCACCATCAT CATCAGCAACAGGTG GCGGCCGCCGCGGCT
EGRX1-3 101  Q  Q  H  Q  Q   Q  Q  Q  Q  Q   H  H  H  H  H   H  Q  Q  Q  V   A  A  A  A  A
CDS     376 GCGTCTTGCATCTGG GGAGCGGTTTACCCG CCGCCGCCGCCCGCG CTCGGATACCATCAC CACCATCACCACCAT
EGRX1-3 126  A  S  C  I  W   G  A  V  Y  P   P  P  P  P  A   L  G  Y  H  H   H  H  H  H  H
CDS     451 CACCATCCACCGCCA CCACCCTCAGGCGAG GACACGCAGTGCGGT ACCGAGGAGATTGTC GCCACCAGCCAGGGA
EGRX1-2 151  H  H  P  P  P   P  P  S  G  E   D  T  Q  C  G   T  E  E  I  V   A  T  S  Q  G
EGRX3   151  H  H  P  P  P   P  P  S  G  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -
CDS     526 GGCGACCAACTCCAG AGGATGACGATGGAC GGCATGGAAGTGGTG GGCTCGCAACCCCAC GCGGCCACCAGCCCG
EGRX1-2 176  G  D  Q  L  Q   R  M  T  M  D   G  M  E  V  V   G  S  Q  P  H   A  A  T  S  P
EGRX3           -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -
CDS     601 TTCTTGCTCTCGCCG CCACCCCTTGGACAC CACCATCATCATCAT CATCCACACGCCACC TTCAACCTGCAAACG
EGRX1-2 201  F  L  L  S  P   P  P  L  G  H   H  H  H  H  H   H  P  H  A  T   F  N  L  Q  T
EGRX3           -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -
CDS     676 GTGCAGCTCAGTTTC GACGCGTGTCTACCG TACAACTCGGCGACG TCCTCGAATTCGATC GTATGCGGCGTTGGC
EGRX1-2 226  V  Q  L  S  F   D  A  C  L  P   Y  N  S  A  T   S  S  N  S  I   V  C  G  V  G
EGRX3           -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -
CDS     751 GGCACGTCAACACCG GCCGCCACCACTTGC ACCTCGTCCACCAGC TGCAACAAGACGATA CCGAGCCTGTCGCTG
EGRX1-2 251  G  T  S  T  P   A  A  T  T  C   T  S  S  T  S   C  N  K  T  I   P  S  L  S  L
EGRX3           -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -
CDS     826 GCCCCTTGCGCGCCC CTGCAAGCCCAGCAT CAAAACAGTGCCGAT ACCACGGATCATCAT GGCCGGCATCATCAA
EGRX1-2 276  A  P  C  A  P   L  Q  A  Q  H   Q  N  S  A  D   T  T  D  H  H   G  R  H  H  Q
EGRX3           -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -
CDS     901 CACCACCATCAACGG CTCAACGATCACCAG CATCACCGGCACGTC GACGCTTCCTCGCCC TCCAGCGACTCGACC
EGRX1-2 301  H  H  H  Q  R   L  N  D  H  Q   H  H  R  H  V   D  A  S  S  P   S  S  D  S  T
EGRX3           -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -
CDS     976 GACGGGAAACGACGT TTGCAGTCCGTCAAC GAGGAGAAGGACTGC TCCAGGGAGTGCAGA GACGATCTTCAGGAG
EGRX1-2 326  D  G  K  R  R   L  Q  S  V  N   E  E  K  D  C   S  R  E  C  R   D  D  L  Q  E
EGRX3           -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -
CDS    1051 TCTAACGATAAGGAT AAAACAGGCGACCTA AACACGCCGGTTACC ACCAGCAGCGATTTA CCGTCCTTCTTCGGC
EGRX1   351  S  N  D  K  D   K  T  G  D  L   N  T  P  V  T   T  S  S  D  L   P  S  F  F  G
EGRX2   351  S  N  D  K  D   K  T  G  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -
EGRX3           -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -
CDS    1126 CCCTCCGCGCTCGTC GAGCCACCTCCTATT TCAGGCAGCCTGGCG GGCGAAGACCTATCC CTGGAAGAGGCGACC
EGRX1   376  P  S  A  L  V   E  P  P  P  I   S  G  S  L  A   G  E  D  L  S   L  E  E  A  T
EGRX2   359  -  -  -  -  -   -  -  -  -  -   -  -  S  L  A   G  E  D  L  S   L  E  E  A  T
EGRX3   160  -  -  -  -  -   -  -  -  -  -   -  -  S  L  A   G  E  D  L  S   L  E  E  A  T
CDS    1201 GCGGAGGACGATGAA ACGGGTACGTCGACG GGCAGGGATCATCGA CACCACCATCACCAA GAGTCTCAAGATCAA
EGRX1-3 401  A  E  D  D  E   T  G  T  S  T   G  R  D  H  R   H  H  H  H  Q   E  S  Q  D  Q
CDS    1276 GGAACGCCTGGCGCC CCTCCCTCCGCTAGT CCGCCGCGTCACCAC CAAGCCCAAGACGAC GCGGACCGTTGCAAC
EGRX1-3 426  G  T  P  G  A   P  P  S  A  S   P  P  R  H  H   Q  A  Q  D  D   A  D  R  C  N
CDS    1351 GTGTTGCAAGGCGGC GTGATCCTCTACTCG CCCCATTCCACCTCC TCGGTGTCCTCGGCG CCCGCCTCCAGCGTC
EGRX1-3 451  V  L  Q  G  G   V  I  L  Y  S   P  H  S  T  S   S  V  S  S  A   P  A  S  S  V
CDS    1426 AACATCTGCAACGTG CCCACCCTCACCTAC CACGGGGTCTTCACC ACCACCTGCACCCAG TCCTCGCCCCTGAAC
EGRX1-3 476  N  I  C  N  V   P  T  L  T  Y   H  G  V  F  T   T  T  C  T  Q   S  S  P  L  N
CDS    1501 GCTCAGAACCAACAG CAGCCGCCCCAGCAG CAGCAGCAGCAGCAG CAGCAGCAGCCGACC ACCCAGGAGCTCTGG
EGRX1-3 501  A  Q  N  Q  Q   Q  P  P  Q  Q   Q  Q  Q  Q  Q   Q  Q  Q  P  T   T  Q  E  L  W
CDS    1576 GGTCCGTTGACCTCC CCCACCCTGACCTTG ACGAATCCGCCGTTC CTCCACTCGGCGCTC CACTCGGCCCCGTAC
EGRX1-3 526  G  P  L  T  S   P  T  L  T  L   T  N  P  P  F   L  H  S  A  L   H  S  A  P  Y
CDS    1651 GGTGGCGAGACCGTC GAACTTCTACCCGTC GAGTCGAAGCCACCC CCGCCACCAGGTTAC CATGACACGCCGACC
EGRX1-3 551  G  G  E  T  V   E  L  L  P  V   E  S  K  P  P   P  P  P  G  Y   H  D  T  P  T
CDS    1726 ACGACCCAGGCCGCG TGGCTGACCACGCAC GAGGACCCCTACGAT CCGAATCTCCTGTCC CACCATCATCCCCAT
EGRX1-3 576  T  T  Q  A  A   W  L  T  T  H   E  D  P  Y  D   P  N  L  L  S   H  H  H  P  H
CDS    1801 CACCACCGCCAAGAG GCCGCGTTGAAGCAG GAGCCTAGCGGGGCG AGCGGTTACGGGCCG GCCGTTCAACAGCAG
EGRX1-3 601  H  H  R  Q  E   A  A  L  K  Q   E  P  S  G  A   S  G  Y  G  P   A  V  Q  Q  Q
CDS    1876 CAGCAGCAACAGCCG CAGCAAGCTCAGCCG AGCCAGCAGCAGCAG CAGCAGCAACAGCAA CCACCACCACCGTCT
EGRX1-3 626  Q  Q  Q  Q  P   Q  Q  A  Q  P   S  Q  Q  Q  Q   Q  Q  Q  Q  Q   P  P  P  P  S
CDS    1951 GCGGGTACGACGGGC GTCCAGCTGGCCGAG TACAATCCGAGCACG TCGAAGGGACACGAG ATTCTCTCGCAAGTT
EGRX1-3 651  A  G  T  T  G   V  Q  L  A  E   Y  N  P  S  T   S  K  G  H  E   I  L  S  Q  V
CDS    2026 TACCAGCAGAGCCCT CTGCCGCTCAGGCTG GTCCCGGTGAAGCCG CGCAAGTACCCGAAT CGACCGAGCAAAACG
EGRX1-3 676  Y  Q  Q  S  P   L  P  L  R  L   V  P  V  K  P   R  K  Y  P  N   R  P  S  K  T
CDS    2101 CCGGTGCACGAGCGG CCGTACGCTTGCCCC GTGGACGGTTGCGAC CGCAGGTTCTCCCGC AGCGACGAGCTCACC
EGRX1-3 701  P  V  H  E  R   P  Y  A  C  P   V  D  G  C  D   R  R  F  S  R   S  D  E  L  T
CDS    2176 CGGCACATCCGCATC CACACGGGCCAGAAG CCGTTCCAGTGTCGC ATCTGCATGCGCTCG TTCTCGAGGAGCGAC
EGRX1-3 726  R  H  I  R  I   H  T  G  Q  K   P  F  Q  C  R   I  C  M  R  S   F  S  R  S  D
CDS    2251 CACCTCACCACCCAC GTGAGAACGCACACC GGCGAGAAACCGTTC TGCTGTGATCAATGC GGCCGAAAGTTCGCG
EGRX1-3 751  H  L  T  T  H   V  R  T  H  T   G  E  K  P  F   C  C  D  Q  C   G  R  K  F  A
CDS    2326 AGGAGCGACGAGAAG AAGCGGCACGCCAAA GTCCACCTCAAGCAG AGGCTGAAGCGCGAG GCCACCCACGCCTCG
EGRX1-3 776  R  S  D  E  K   K  R  H  A  K   V  H  L  K  Q   R  L  K  R  E   A  T  H  A  S
CDS    2401 GCGAGAAATCACCCT CAGAGCCACGCTTCG CCTCCTTGCAACCAG TAA
EGRX1   801 A  R  N  H  P   Q  S  H  A  S   P  P  C  N  Q   *    815
EGRX2   772 A  R  N  H  P   Q  S  H  A  S   P  P  C  N  Q   *    786
EGRX3   573 A  R  N  H  P   Q  S  H  A  S   P  P  C  N  Q   *    587
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Fig 11 *Coding DNA sequence (CDS) of the Amegr gene and translated protein sequences of the aligned isoforms (EGRX1-3)*. Highlighted DNA sequences represent location of forward primers specifically designed to detect *AmegrX1* (dark blue; primer X1_b), *AmegrX2* (mid blue), and *AmegrX3* (light blue). Note that all three primers share the target triplicate at the nucleotide position 1162-1164 (start of exon 3, compare with **Fig 9** and **Fig 12**). In turquoise, the position of the forward primer used in *Chapter III* (primer X1_a) is indicated. The position of the shared reverse primer is marked in magenta (actual primer sequence is reverse complement to DNA sequence, see **Table 3**). The green labeled amino acid sequence indicates the peptide used for immunization and antibody synthesis, i.e. the protein region that is detected by the applied antibodies. Highlighted in gray are the three structural motifs of the zinc finger DNA binding sites (see **Fig 10**). Vertical dotted lines indicate exon-exon junctions. Data obtained from the NCBI BioSystems database. ▲

in PBS at 100 µm (Leica VT1000S, Leica Biosystems, Wetzlar, Germany). Thereafter, the sections were washed in 2% Triton-X100 in PBS (1x10 min), then in 0.2% Triton-X100 in PBS (2x10 min), and pre-incubated in 0.2% Triton-X100 in PBS with 2% normal goat serum (NGS) for 1h at room temperature. In the next step, slices were incubated with the primary antibodies directed against AmEGR (0.1 µg/ml in PBS with 0.2% Triton-X100 and 2% NGS) for two days at 4°C. After washing in PBS (5x10 min), brain slices were incubated with the fluorochrome-associated secondary antibody (Alexa488@rabbit; 1:250 in PBS with 1% NGS) and labelled f-actin-binding phalloidin (CF633; 1:200) over night at 4°C. Three times washing in PBS (á 10 min) was followed by a 30 min incubation with HOECHST (1:1000 in PBS) to label the nuclei. After a final washing step with PBS (3x10 min), the slices were transferred to 60% glycerol in PBS for 30 min and eventually mounted in 80% glycerol in PBS on microscope slides, sealed with nail polish and stored at -20°C.

To validate the specificity of the anti-AmEGR antibody, an additional peptide competition assay (PCA) was performed. Therefore, the peptide used for immunization and antibody production was dissolved in PBS (concentration of 10 mg/ml), and the anti-AmEGR antibody was pre-incubated with the peptide (mole-proportion of 1:100 antibody:peptide; 1.8 µl of anti-AmEGR to 125 µl of peptide in PBS) over night. The pre-incubated antibody was used for immunostaining as described above.

Brain slices were analyzed using a laser scanning confocal microscope (Leica TCS SP2 AOBS, Leica Microsystems, Wetzlar, Germany). Images were taken at a resolution of 1,024 x 1,024

pixels using two different objectives with additional digital zoom: 10x/0.4 NA for images of central brain and optic lobes, and 20x/0.7 NA for images of calyces and antennal lobes.

Statistics and software

Difference in learning performance between paired and unpaired group was calculated using Mann-Whitney-U test. Expression values of isoforms were compared among treatment groups using Kruskal-Wallis test with subsequent post hoc pairwise analysis (MWU). All calculations were performed with IBM® SPSS® Statistics software (Version 20).

Results

When selected for visual conditioning, the group of bees trained with a paired presentation of the CS and US quickly learned the association. 35% of all paired-conditioned bees showed a conditioned response (PER in response to the CS alone) in two or more training trials. Of these individuals, 86% exhibited the response after five trials (**Fig 13**). In contrast, not a single bee trained with an unpaired presentation of the stimuli responded to the monochromatic light by extending the proboscis. The difference in learning performance between the paired and the unpaired group was highly significant ($p<0.001$, $Z=-8.171$). Individual bees within experimental groups were then randomly assigned for brain dissection at one of three different time points: 30 (equivalent to the moment immediately after end of conditioning),

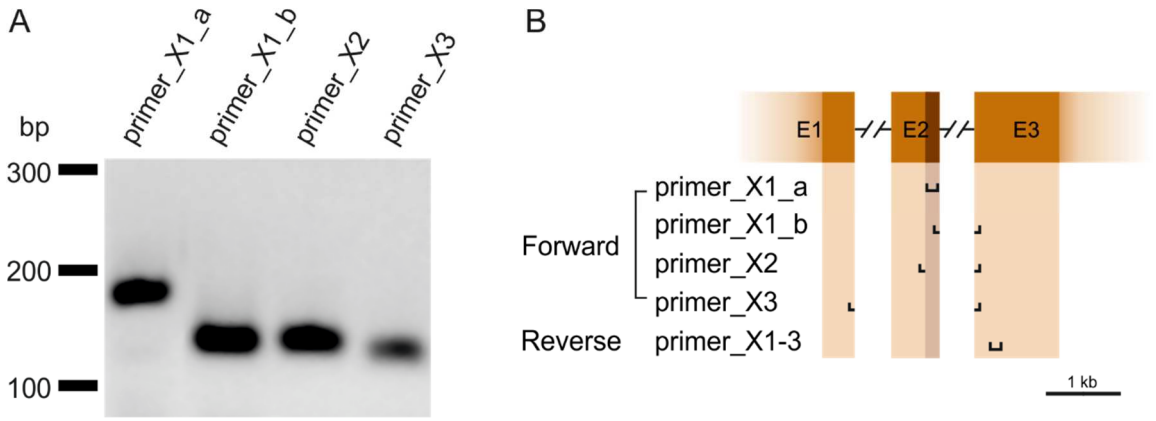

Fig 12 *Isoform-specific oligonucleotide primers targeting Amegr transcripts*. **A** Validation of primers by means of agarose gel electrophoresis of PCR products. The applied forward primers are given above the gel lanes. **B** Location of target sequences of the isoform-specific primers (compare with **Fig 9** and see **Table 3**). Different forward primers were used to detect single isoforms, while the same reverse primer was utilized for all isoforms. E1-E3, exon 1-3.

90 and 240 min after the first CS-US association. Analysis of cerebral expression of *Amegr* isoforms in the mushroom bodies revealed that, independently of the treatment group, the expression level decreased over time and was, in most groups, lowest at 240 min after the first CS-US association. For *AmegrX1* (**Fig 14**), no learning-dependent effect was observed, i.e. no differences occurred among treatment groups within a specific time point of sampling, whereas for all groups the level of *AmegrX1* was reduced 240 min after start of behavioral treatment (for statistics of post hoc pairwise comparison see **Table 4**). Targeting the second isoform, *AmegrX2*, revealed comparable results (**Fig 15**): no differences were found among treatment groups, but in all groups, the level of *AmegrX2* was decreased at the last sampling point. While this reduction was significant for the naïve and paired group, only a trend was observable in the unpaired group (statistics in **Table 4**). The expression pattern of the third isoform, *AmegrX3*, was less distinct and a significant reduction was only measured in the paired conditioning group at 240 min after start of training, when compared with the naïve group (0-90 min) and the first sampling point of the paired group (**Fig 16** and **Table 4**).

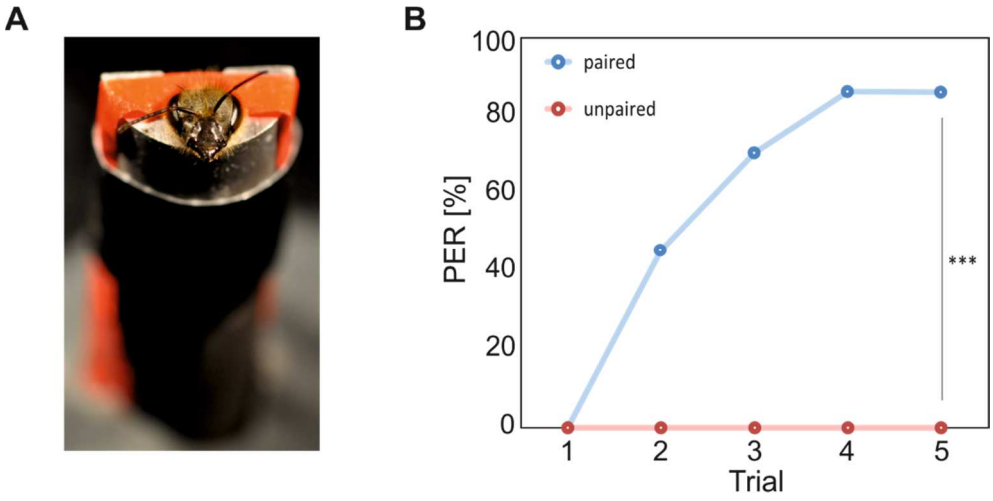

Fig 13 *Visual conditioning of the PER in honeybees*. **A** A harnessed bee in a metal holder, prepared for conditioning experiments. **B** Population learning curve: proportion of bees exhibiting the extension of the proboscis (PER) per learning trial. Bees received either a paired (blue) or an unpaired (red) presentation of the CS (blue light) and US (sucrose solution) to form an association. paired, N=44; unpaired, N=37

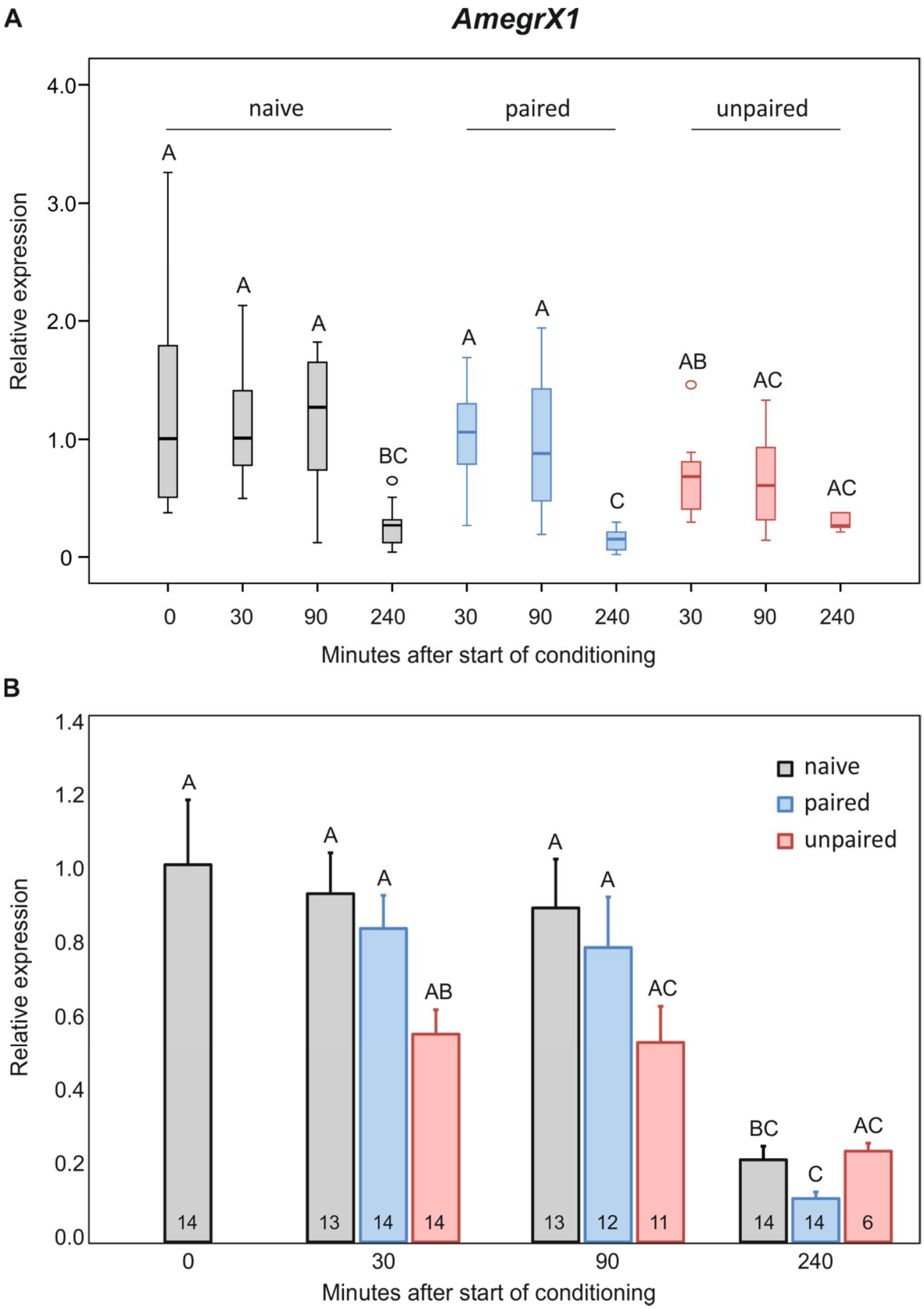

Fig 14 *Expression of AmegrX1 in the mushroom body calyces of visually trained and control honeybees at different time points*. Data for *AmegrX1* expression are shown as box plots (**A**) and histogram (**B**, mean ±SEM). Same letters indicate no significant difference. Numbers in bars represent sample size. Kruskal-Wallis: $p<0.001$; $chi^2=66.872$; pairwise comparison: see **Table 4**

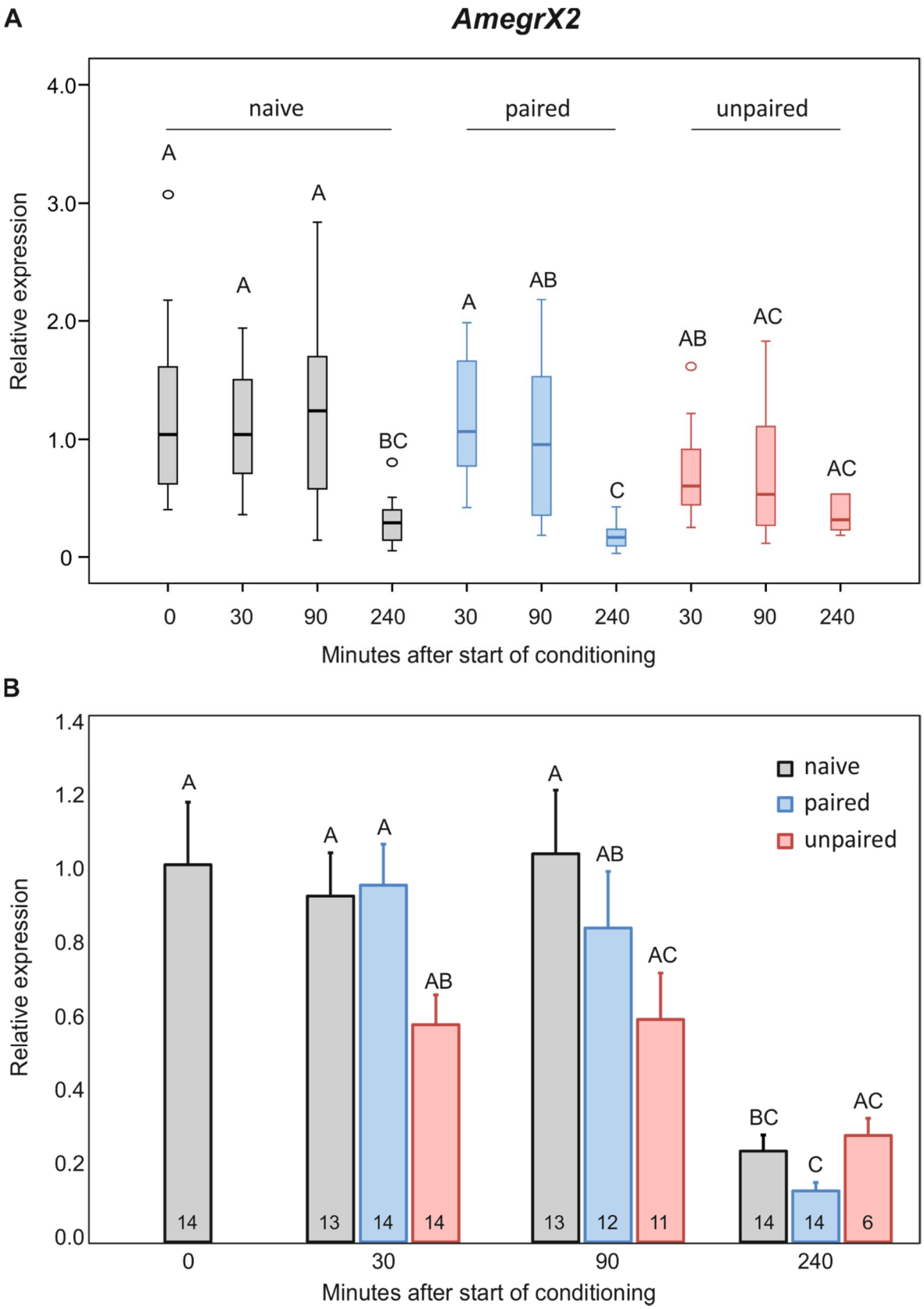

Fig 15 *Expression of AmegrX2 in the mushroom body calyces of visually trained and control honeybees at different time points*. Data for *AmegrX2* expression are shown as box plots (**A**) and histogram (**B**, mean ±SEM). Same letters indicate no significant difference. Numbers in bars represent sample size. Kruskal-Wallis: $p<0.001$; $chi^2=60.100$; pairwise comparison: see **Table 4**

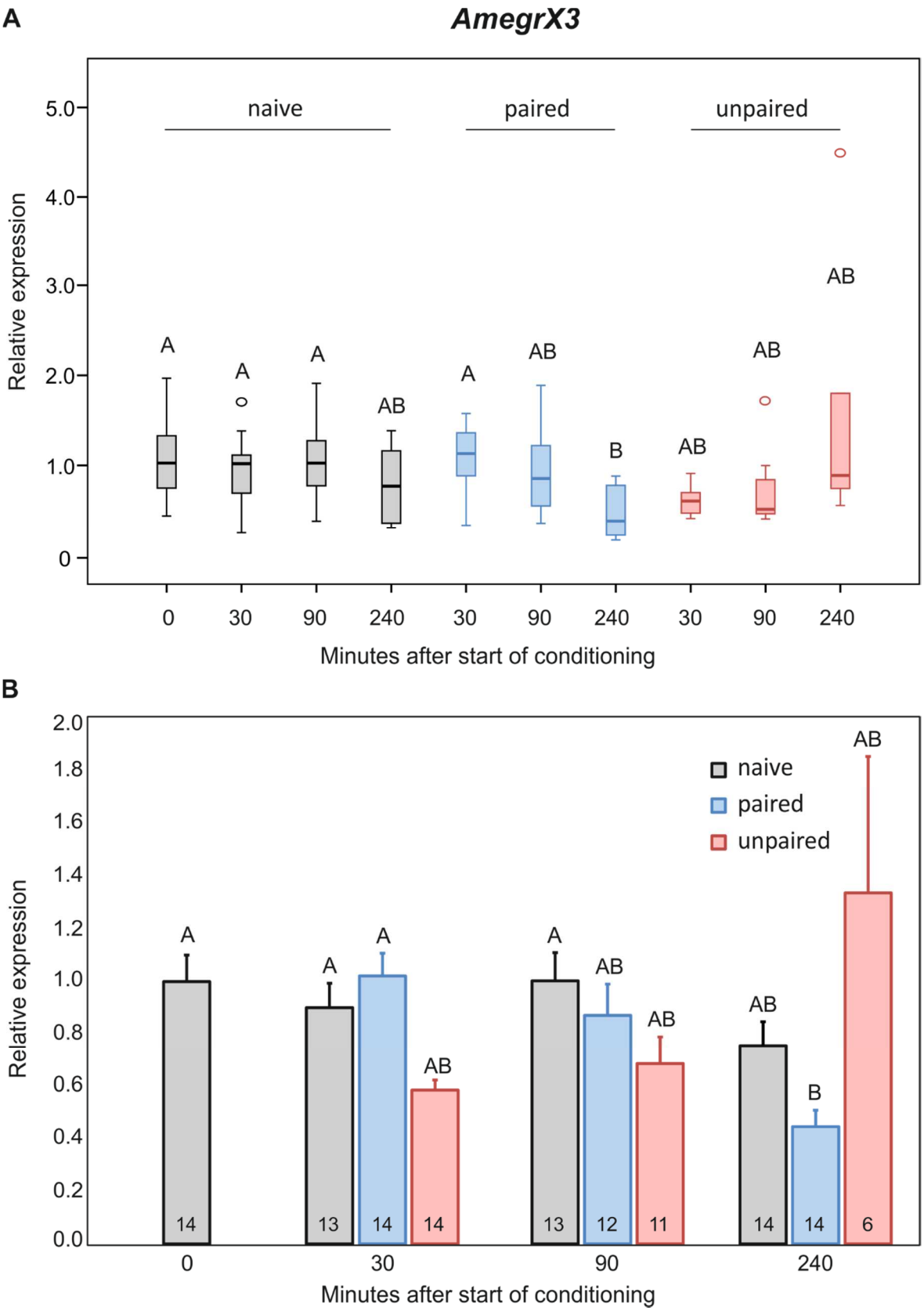

Fig 16 *Expression of AmegrX3 in the mushroom body calyces of visually trained and control honeybees at different time points*. Data for *AmegrX3* expression are shown as box plots (**A**) and histogram (**B**, mean ±SEM). Same letters indicate no significant difference. Numbers in bars represent sample size. Kruskal-Wallis: $p<0.001$; $chi^2=35.882$; pairwise comparison: see **Table 4**

	unpaired_240	unpaired_90	unpaired_30	paired_240	paired_90	paired_30	naive_240	naive_90	naive_30	naive_0
naive_0	p=0.080 Z=3.125	p=1.000 Z=1.892	p=1.000 Z=1.664	p<0.001 Z=5.374	p=1.000 Z=0.827	p=1.000 Z=0.219	p=0.001 Z=4.294	p=1.000 Z=0.273	p=1.000 Z=-0.171	
naive_30	p=0.057 Z=3.223	p=1.000 Z=2.022	p=1.000 Z=1.804	p<0.001 Z=5.444	p=1.000 Z=0.977	p=1.000 Z=0.386	p=0.001 Z=4.385	p=1.000 Z=0.436		p=1.000 Z=-0.012
naive_90	p=0.181 Z=2.877	p=1.000 Z=1.605	p=1.000 Z=1.361	p<0.001 Z=5.001	p=1.000 Z=0.550	p=1.000 Z=-0.057	p=0.004 Z=3.941		p=1.000 Z=0.019	p=1.000 Z=0.007
naive_240	p=1.000 Z=-0.201	p=1.000 Z=-2.136	p=0.385 Z=-2.629	p=1.000 Z=1.080	p=0.044 Z=-3.299	p=0.002 Z=-4.074		p=0.004 Z=3.927	p=0.004 Z=3.947	p=0.003 Z=4.009
paired_30	p=0.141 Z=2.955	p=1.000 Z=1.687	p=1.000 Z=1.445	p<0.001 Z=5.154	p=1.000 Z=0.616		p=0.001 Z=-4.199	p=1.000 Z=-0.194	p=1.000 Z=-0.174	p=1.000 Z=-0.190
paired_90	p=0.739 Z=2.399	p=1.000 Z=1.047	p=1.000 Z=0.772	p=0.001 Z=4.336		p=1.000 Z=0.847	p=0.064 Z=-3.188	p=1.000 Z=0.646	p=1.000 Z=0.664	p=1.000 Z=0.664
paired_240	p=1.000 Z=-1.037	p=0.074 Z=-3.149	p=0.009 Z=-3.709		p=0.002 Z=4.120	p<0.001 Z=5.170	p=1.000 Z=0.970	p<0.001 Z=4.879	p<0.001 Z=4.899	p<0.001 Z=4.979
unpaired_30	p=1.000 Z=1.836	p=1.000 Z=0.331		p=0.026 Z=-3.446	p=1.000 Z=0.810	p=1.000 Z=1.724	p=0.599 Z=-2.475	p=1.000 Z=1.498	p=1.000 Z=1.517	p=1.000 Z=1.534
unpaired_90	p=1.000 Z=1.502		p=1.000 Z=0.276	p=0.140 Z=-2.957	p=1.000 Z=1.029	p=1.000 Z=1.893	p=1.000 Z=-2.046	p=1.000 Z=1.680	p=1.000 Z=1.698	p=1.000 Z=1.714
unpaired_240		p=1.000 Z=1.250	p=1.000 Z=1.527	p=1.000 Z=-1.142	p=1.000 Z=2.128	p=0.189 Z=2.863	p=1.000 Z=-0.390	p=0.332 Z=2.679	p=0.317 Z=2.694	p=0.298 Z=2.694
naive_0	p=1.000 Z=0.527	p=1.000 Z=2.202	p=0.185 Z=2.870	p=0.002 Z=4.041	p=1.000 Z=0.975	p=1.000 Z=-0.337	p=1.000 Z=1.591	p=1.000 Z=-0.037	p=1.000 Z=0.391	
naive_30	p=1.000 Z=0.216	p=1.000 Z=1.799	p=0.688 Z=2.425	p=0.016 Z=3.575	p=1.000 Z=0.582	p=1.000 Z=-0.721	p=1.000 Z=1.171	p=1.000 Z=-0.420		
naive_90	p=1.000 Z=0.549	p=1.000 Z=2.201	p=0.195 Z=2.853	p=0.003 Z=4.002	p=1.000 Z=0.993	p=1.000 Z=-0.294	p=1.000 Z=1.598			
naive_240	p=1.000 Z=-0.706	p=1.000 Z=0.710	p=1.000 Z=1.278	p=0.644 Z=2.450	p=1.000 Z=-0.554	p=1.000 Z=-1.928				
paired_30	p=1.000 Z=0.787	p=0.531 Z=2.518	p=0.061 Z=3.206	p=0.001 Z=4.377	p=1.000 Z=1.298					
paired_90	p=1.000 Z=-0.253	p=1.000 Z=1.207	p=1.000 Z=1.782	p=0.164 Z=2.907						
paired_240	p=0.415 Z=-2.603	p=1.000 Z=-1.588	p=1.000 Z=-1.171							
unpaired_30	p=1.000 Z=-1.696	p=1.000 Z=-0.489								
unpaired_90	p=1.000 Z=-1.242									

AmegrX1
AmegrX2
AmegrX3

Table 4 *Statistics for post hoc pairwise comparison*. Values for *AmegrX1* (blue), *AmegrX2* (black) and *AmegrX3* (red)

Spatial distribution of AmEGR protein

To validate the specificity of the anti-AmEGR antibody, Western blot analysis was performed (**Fig 17**). A positive immuno-signal was found for all tested brain compartments. While a (partially weak) signal of a double band matching approx. 90 kDa was detectable in all lysates, another band at around 60 kDa was most prominent in the lysate containing optic lobe tissues. This band was only weakly detectable in mushroom body lysates and completely absent in the

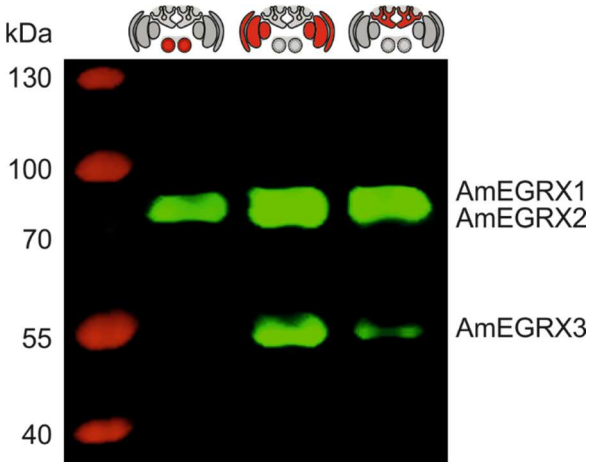

Fig 17 *Antibody validation using Western blot analysis*. The anti-AmEGR antibody was tested on lysates of three different brain compartments including antennal lobes, optic lobes and mushroom bodies (from left to right). First lane shows protein marker (PageRuler^{TM}, ThermoFisher Scientific). The green bands represent immune-reactivity against AmEGR proteins; isoforms are indicated on the right.

antennal lobe lysates. The spatial distribution of the AmEGR signal was then analyzed by immunohistofluorescence imaging of brain sections (**Fig 18** and *Appendix I*). Positive immuno-signal was detectable in many parts of the brain with prominent signals in the calyces, as well as the optic lobes and antennal lobes. In more detail, the staining was exclusively located in the cell nuclei, as indicated by co-localization with HOECHST fluorescence signal (**Fig 19**). The most prominent immuno-signal was observed in cell nuclei of Kenyon cells, the mushroom body intrinsic neurons, primarily located within the cup-shaped calyces. Additional clusters of AmEGR-positive cell nuclei were found between retinotopic neuropils of the optic lobes and in the periphery of the antennal lobes. Overall, no nuclei without AmEGR-positive signal were observed. In contrast, the control assay with anti-AmEGR antibodies pre-incubated with the immunizing peptide revealed no detectable immnunosignal and indicates target-specific binding of the antibody (**Fig 20**).

Discussion

Honeybees quickly learn to associate a color stimulus with a reward (**Fig 13**). While this is long known for free-flying bees (von Frisch 1914, Menzel 1967, von Helversen 1972, Dyer and Chittka 2004a), conditioning of visual stimuli in restraint conditions using the PER assay was successful only recently (Dobrin and Fahrbach 2012, Jernigan *et al.* 2014, Lichtenstein *et al.*

2015). As compared to the results by Dobrin and Fahrbach (2012), honeybees in this study performed equally well in terms of total proportion of learners (bees that responded to the CS prior to US presentation; approx. one third of the tested bees) and group performance of learners after five trials (approx. 80 % PER response).

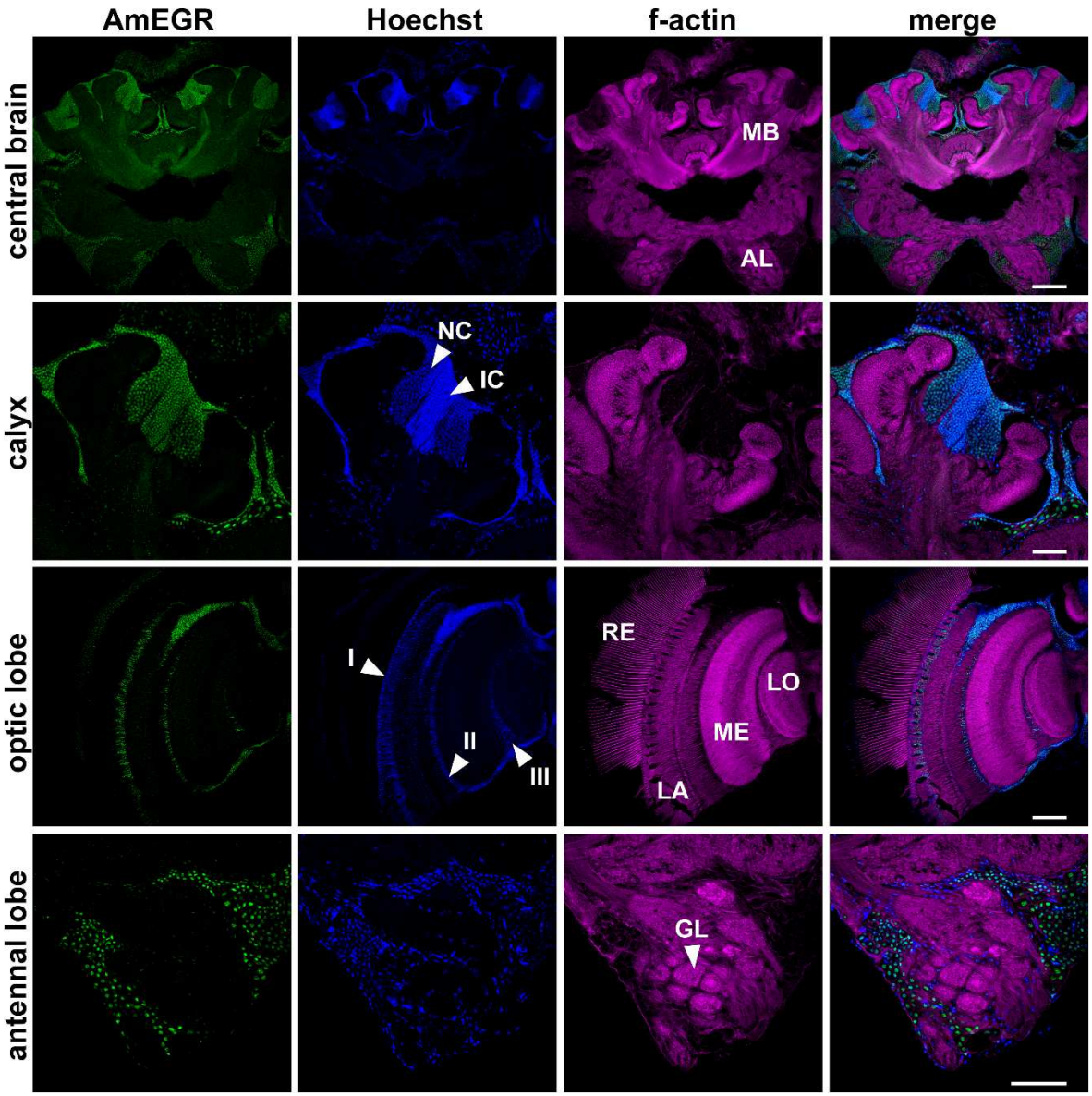

Fig 18 *Immunofluorescence imaging of honeybee brain frontal sections triple-labeled with antibodies directed against AmEGR (green), HOECHST (nuclei, blue), and labeled phalloidin (f-actin, magenta)*. Arrowheads highlight clusters of cell nuclei: NC, IC, non-compact and inner compact cells of Kenyon cells; I, cell body layer of lamina monopolar cells; II, nuclei of trans-medulla and medulla inter neurons; III, cell bodies of lobula intrinsic neurons and optic tract neurons. AL, antennal lobe; MB, mushroom body; RE, retina; LA, lamina; ME, medulla; LO, lobula; GL, glomerulus in antennal lobe. Scale bar: 200 µm (central brain and optic lobes) and 100 µm (calyx and antennal lobes).

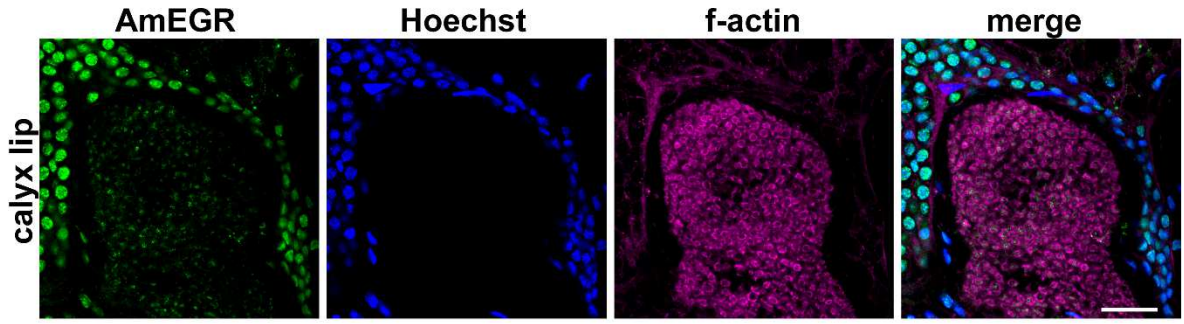

Fig 19 *Magnified image of the calyx lip region triple-labeled for AmEGR (green), cell nuclei (blue) and f-actin (magenta)*. Close inspection reveals strong co-localization of AmEGR with HOECHST fluorescence signal within the nuclei. Scalce bar: 25 µm

The visual conditioning itself does not seem to activate the mRNA expression of the *Amegr* isoforms (**Fig 14-16**), even though the group of bees trained to a paired presentation of CS and US showed highest mRNA expression levels of all three isoforms right after conditioning with a significant decline over time and minimum expression four hours after start of conditioning. However, almost the same expression pattern was observed in the naïve control bees, which additionally possessed high *AmegrX1-X3* levels at time point zero, i.e. before conditioning started. Thus, no expression differences were induced by conditioning. Contrary to this result, Lutz and Robinson (2013) reported an upregulation of *Amegr* in mushroom bodies 30 minutes after orientation flights, which can be considered as a form of visual learning. In their study, *Amegr* mRNA was shown to be enriched in the mushroom bodies as compared to other brain regions (by *in situ* hybridization) and upregulation after orientation flights was dependent on the visual perceptibility of environmental novelty, rather than on motor learning or exercise alone (Lutz and Robinson 2013). Moreover, they report a narrow time window of induction, with elevated levels 30 min after stimulation and a rapid decrease to baseline levels after another 30 min. Besides the issue of using isoform-unspecific primers (as discussed in *Chapter III*), the main difference between the two studies concerns the motoric state of the honeybees: while Lutz and Robinson (2013) tested freely flying/moving bees, in the present study bees were harnessed prior to behavioral testing. Thus, a learning-dependent induction of *Amegr* mRNA may require some sort of motor activity, as it was suggested in mice (Clark *et al.* 2011). The impact of exercise and physical activity on cognition and learning performance is long known in vertebrates (rodents: Radak *et al.* 2001, van Praag *et al.* 2005, humans:

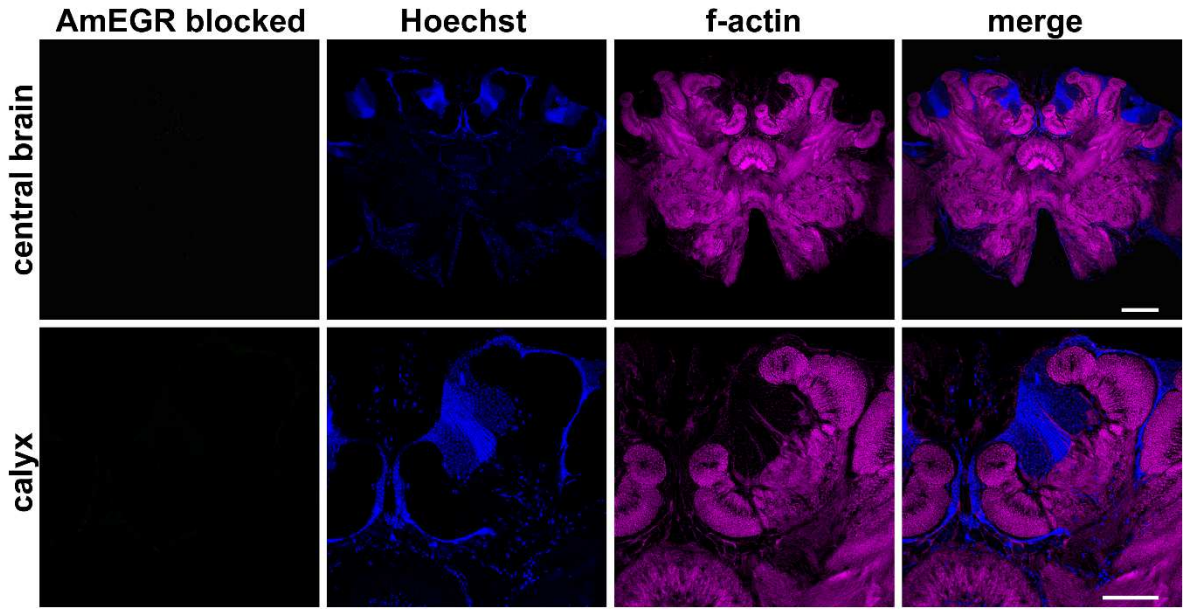

Fig 20 *Peptide competition assay using AmEGR pre-incubated anti-AmEGR antibodies*. Anti-AmEGR antibody blocked by peptide (AmEGR blocked) and regular staining with HOECHST and labeled phalloidin (f-actin). Lack of anti-AmEGR signal indicates target specificity. Scale bar: 200 µm (central brain) and 100 µm (calyx).

Kramer *et al.* 2006), and accordingly, bees perform better (i.e. possessing a higher color discrimination ability) in visual conditioning when trained under free-flight conditions, as compared to restraint conditions (Niggebrugge *et al.* 2009, Lichtenstein *et al.* 2015). Different pathways of color processing, depending on task complexity, are suggested and currently under debate (Dyer *et al.* 2011). Therefore, *egr* could potentially be involved in advanced learning behaviors with concomitant motor activities.

Contrary to this hypothesis are findings by Ugajin *et al.* (2013), who reported an activation of *Amegr* mRNA expression after seizures induced by awakening from CO_2-anestization. Here, no associative learning and advanced motor activity (besides muscular contraction during seizures) precedes the induction. Rather, an unspecific activation of neural cells seems to cause elevated *Amegr* levels. This was affirmed by pharmacological inhibition of $GABA_A$ receptors (through picrotoxin treatment) – mainly affecting the inhibitory GABAergic feedback system projecting into the calyces – that resulted in a three-fold increase of the mRNA (Ugajin *et al.* 2013). Noteworthy, while the expression of *Amegr* after seizure induction is significantly elevated 30 to 60 min after stimulation (Ugajin *et al.* 2013), in orientation flight experiments

the baseline expression level is already reached after one hour (Lutz and Robinson 2013). This discrepancy may suggest multiple roles and activation pathways of *Amegr*.

An alternative explanation for the lack of learning-dependent effects in this study may reside in the conditioning approach. As reported in rodents, restrainment stress can provoke an elevated *egr* expression (Bing *et al.* 1991, Melia *et al.* 1994). In this case, the fixation in conditioning tubes alone could have led to an induction of *Amegr*. This is in accordance with the measured elevated mRNA levels in the naïve control bees at time point zero, compared to the last sampling point four hours later. Thus, an interference of restrainment and learning effects cannot be excluded, even though the bees were allowed to be accustomed to the harnesses overnight and long-term effects of restrainment stress on *egr* expression were absent in rats (Melia *et al.* 1994).

However, some differences in the expression of the three isoforms could be identified. The highest transcription level (indicated by C_T-values) was obtained for *AmegrX1* with a two-fold (*AmegrX2*; C_T-difference of 1 to 1.5) to >30-fold (*AmegrX3*; C_T-difference of 5 to 6) expression compared to the other two isoforms. The observed temporal expression pattern varied only slightly among isoforms, independently from treatment group. While *AmegrX1* and *AmegrX2* both possessed constant expression levels from time point zero to 90 min after start of conditioning, 150 min later (240 min after start of conditioning) a considerable decrease in mRNA levels was measured (**Fig 14** and **15**). In contrast, the level of *AmegrX3* mRNA remained more constant over time (with exceptions in the paired group; **Fig 16**). These differences in mRNA expression patterns may reflect variations in the functional role of the isoforms. In vertebrates, distinct functions of the EGR family members in synaptic plasticity and memory formation are known and range from involvements in processes of early-LTP and STM (Egr3; Li *et al.* 2007) over late-LTP and LTM (Egr1; Jones *et al.* 2001), to regulation of brain segmentation and nerve myelination (Egr2; Schneider-Maunoury *et al.* 1993, Topliko *et al.* 1994, reviewed in Poirier *et al.* 2008). Moreover, functional differences have been proposed for single isoforms of Egr3, as they differ in their abilities to activate transcription (O'Donovan *et al.* 2000), even though they target the identical, EGR-specific consensus sequence. Thus, potential different functions of the three *Amegr* isoforms in memory consolidation need to be further investigated with a methodological trade-off between free-flight and laboratory conditions, considering the issues of natural-like behavior and controlled environmental conditions, respectively.

Analysis of immunofluorescence imaging of AmEGR protein distribution revealed positive signal in apparently all nuclei of the brain cells, indicated by co-localization with HOECHST fluorescence signal. The most prominent fluorescence signal was detectable in the mushroom body calyces (**Fig 18** and *Appendix I*), probably due to the high number of densely packed Kenyon cells (as described in *Chapter V*). Additionally, the cell body layers of the optic lobes and antennal lobes showed comprehensive AmEGR-positive intra-nuclear immune-reactivity. This subcellular pattern of protein distribution corresponds to the observed nuclear accumulation of Egr-1 in stimulated rat cells (Aggeli *et al.* 2010). Considering its function as a transcription regulating factor, the protein's intended site of action is the nucleus. However, translation of transcription factor-encoding mRNA occurs in the cell's cytoplasm, before the functional protein is translocated to the nucleus. Here, a certain reservoir of EGR seems to be stored in the perinuclear space and upon stimulation (e.g. mechanical strain stress), the protein gets uniformly dispersed in the nucleus (Morawietz *et al.* 1999). Besides the translocation of stored protein into the nucleus, cellular stimulation also results in a pronounced synthesis of new EGR protein, reaching a peak level 60 min after stimulation (Morawietz *et al.* 1999, Zangenehpour and Chaudhuri 2002). Thus, the induction of protein expression in response to an incoming stimulation occurs with a delay of 30 min as compared to the encoding mRNA (Clayton 2000, Zangenehpour and Chaudhuri 2002). Whether this temporal expression pattern of AmEGR protein can also be observed in the honeybee remains unresolved.

Altogether, the present study provides the first data on isoform-specific mRNA expression patterns of the honeybee's *Amegr* gene, with temporal analysis of stimulus-dependent differential expression and an exploration of the spatial distribution of the AmEGR protein. Thus, this work aims to provide a valuable starting point for future research on the validation of this IEG as a marker tool for neural activation.

Chapter V – Color and the calyx: does fine color discrimination learning in free-flying honeybees change mushroom body calyx neuroarchitecture?

This chapter was largely adapted for the following manuscript: Sommerlandt FMJ, Spaethe J. Rössler W, Dyer AG. Does fine color discrimination learning in free-flying honeybees change mushroom body calyx neuroarchitecture?

Introduction

Bees are important pollinators of flowers and, in return, flowers often provide a vital source of nutrients for bees (Proctor and Yeo 1972, Barth 1985). Besides olfactory cues (Reinhard *et al.* 2004), bees use a variety of visual information (Dyer *et al.* 2011, Hempel de Ibarra *et al.* 2014) to find rewarding flowers. However, in complex natural environments not all plants present flowers that are rewarding and some flowers mimic truly rewarding flowers to incidentally receive flower visits by insects to facilitate pollination (Dafni 1984, Jersakova *et al.* 2006). This complex foraging situation places demands on the visual processing of bees for fine discriminations (Avargues-Weber and Giurfa 2014), but to date there is a relative dearth of information about how the sensory processing system of bees facilitates such rich visual capabilities as have been observed in psychophysical studies.

Color is one of the most important features used by honeybees to identify flowers as potential food sources (von Frisch 1914, Daumer 1956, von Helversen 1972, Neumeyer 1981, Chittka and Menzel 1992, Giurfa 2004, Dyer and Arikawa 2014). To enable highly efficient foraging, bees not only have to perceive the color information, but also have to learn this information (von Frisch 1965, Menzel 1967) and retrieve it after days or even weeks (Dyer and Garcia 2014). Honeybees are able to learn colors within their perceptual range (from 300 nm to 650 nm), although with varying efficiencies depending upon wavelength (Menzel 1967, von Helversen 1972, reviewed in Giurfa 2003). However, the performance level when discriminating two colors is highly dependent on the way in which stimuli are encountered in a foraging situation (Avargues-Weber and Giurfa 2014). Discrimination of perceptually similar colors requires differential conditioning with target and distractor stimuli. In contrast, when target colors are learnt in isolation with absolute conditioning only a coarse level of color discrimination develops (Dyer and Chittka 2004a, Giurfa 2004). This not only suggests different

levels of behavioral plasticity in bee color learning, but also different underlying neuronal processes. A first step towards understanding these mechanisms is to identify the neuropils where such visual information may be processed and stored in a bee brain. Due to the complexity of visual computations, several brain regions might be involved, either in parallel or via serial processing of such information (Ehmer and Gronenberg 2002, Paulk and Gronenberg 2008, Dyer *et al.* 2011, Mota *et al.* 2013).

Potentially essential neuropils are the paired mushroom bodies (MB) which have been identified as sensory integration centers that facilitate associative learning and (long-term) memory formation (Strausfeld *et al.* 1998, Menzel and Giurfa 2001, Fahrbach 2006, Giurfa 2007, Hourcade *et al.* 2010, Groh and Rössler 2011, Falibene *et al.* 2015). In honeybees, the MBs comprise a high number of neurons (ca. 170,000-184,000 Kenyon cells per MB (Witthöft 1967); reviewed in (Fahrbach 2006, Rössler and Groh 2012)) and take up a large part of the brain volume compared to other neuropils. Recent studies have shown that age, behavior and social environment may affect volumetric properties of the MB and its substructures (Maleszka *et al.* 2009, Groh *et al.* 2012, Muenz *et al.* 2015). The four cup-shaped calyces (one median and lateral calyx per MB and brain hemisphere) represent the sensory input regions of the MBs. These structures are sub-divided into three modality-specific compartments comprising (i) the lip, receiving olfactory information from the antennal lobes, (ii) the collar, receiving visual information from the optical lobes, and (iii) the basal ring, a region that integrates olfactory and visual information (Mobbs 1982, Abel *et al.* 2001, Gronenberg 2001, Ehmer and Gronenberg 2002, Kirschner *et al.* 2006). Neuronal circuits within a calyx are organized in distinct microglomeruli (MG), synaptic complexes consisting of a single presynaptic bouton from the axon terminals of a projection neuron that is surrounded by numerous postsynaptic dendritic spines of MB intrinsic neurons, the Kenyon cells (Groh *et al.* 2004, Groh *et al.* 2006, Groh and Rössler 2011, Rössler and Groh 2012). The MG synaptic circuits are characterized by a high degree of structural plasticity, as changes in the distribution (or density) of the synaptic complexes are found to be associated with age (Groh *et al.* 2012, Muenz *et al.* 2015), light exposure (Scholl *et al.* 2014), and the formation of olfactory long-term memory (Hourcade *et al.* 2010). The stable late form of olfactory long-term memory lasts for 2 days up to lifetime and depends on protein synthesis (Wüstenberg *et al.* 1998, Menzel 1999). This was shown to be accompanied by an increase in MG densities

and total numbers in the olfactory lip region of the MB calyx (Hourcade *et al.* 2010, Falibene *et al.* 2015).

It is unknown whether fine color discrimination and the formation of visual long-term memory is also processed in the MBs and, thus, might affect (or is affected by) the MB calyx neuronal network and synaptic structure. In bees, the visual collar region of the MB calyces is innervated by projection neurons deriving from inner medulla and inner lobula layers (Paulk *et al.* 2009b, reviewed in Dyer *et al.* 2011). Thus, major effects in visual memory formation should take place in the visually innervated collar, although little is known for color learning in free flying bees. Based on this assumption, the present study aims to test the following two hypotheses: First, in analogy to findings in olfactory learning experiments (Hourcade *et al.* 2010), it is proposed that the formation of a new visual memory should be associated with an increase in MG density in the MB collar. Second, the strength of the effect on MG density should be correlated with the complexity of the visual learning task, i.e. compared to an easy absolute conditioning task, bees that learn to discriminate between a pair of perceptually similar colors in a differential conditioning paradigm have to learn more stimulus features, and thus more neuronal circuits may be involved. To investigate whether the MG synaptic network is shaped by visual learning and depends on the level of complexity of a learning task, honeybee foragers were individually trained in either absolute or differential conditioning with two perceptually similar colors and subsequently tested for color discrimination abilities in a choice test. MB characteristics (volume and MG number and density) were measured after three days (to allow long-term memory formation) and tested for potential correlation with behavioral performance. This study aims to provide a first step towards understanding potential neuronal mechanisms underlying color learning and memory formation in bees under natural free-flying conditions.

Material and methods

Behavioral Color Conditioning

Experiments were conducted with the European honeybee (*Apis mellifera carnica*) maintained in a colony located at the Campus Hubland Nord of the Julius Maximilian University (Würzburg, Germany). A feeding site (gravity feeder; e.g. as shown in Spaethe *et al.* 2014) was positioned 25 m away from the colony, from which foragers were allowed to collect 5-10% (w/w) sucrose solution *ad libitum*. Individual bees were transferred (by means of a Plexiglas® spoon with a

drop of sucrose solution) from the feeding site to a test site 6 m away, where they received 25% sucrose solution and were individually color marked on their thorax. Each bee was trained and tested individually, which lasted approx. 90-120 min per individual.

Color stimuli were made from cardboard (6 x 8 cm2; Tonpapier no. 32 [turquoise] and 37 [blue], Baehr, Germany; as used in (Dyer and Garcia 2014, Dyer *et al.* 2014) that appear to a human observer turquoise and blue, respectively (**Fig 21**). The stimuli were covered with matt lamination foil (ARGO SA, 80-393 Gdansk, Poland) and attached to freely rotating hangers with a landing platform, presented on a vertical, circular and rotatable plastic screen of 50 cm diameter (as described in Morawetz *et al.* 2013; in the following referred to as "rotating screen"). This set-up allowed an efficient rearrangement of the stimuli to avoid location learning, and hanger replacement to avoid olfactory markings (Dyer *et al.* 2005). The spectral reflectance of the stimuli was measured with a JAZ S1 spectrometer (Ocean Optics; **Fig 21A**) and color loci were calculated in a hexagon color space (Chittka 1992; **Fig 21B**). The color distance (considering the grey background color of the rotating screen) between turquoise and blue was 0.075 hexagon units, which is sufficiently large to be discriminated by bees (Dyer and Chittka 2004b, Dyer and Neumeyer 2005). Target stimuli (CS+) were reinforced by 10 µl of 25% sucrose solution (US+) placed on the landing platform, whereas distractor stimuli (CS-) contained 10 µl of pure water (US-).

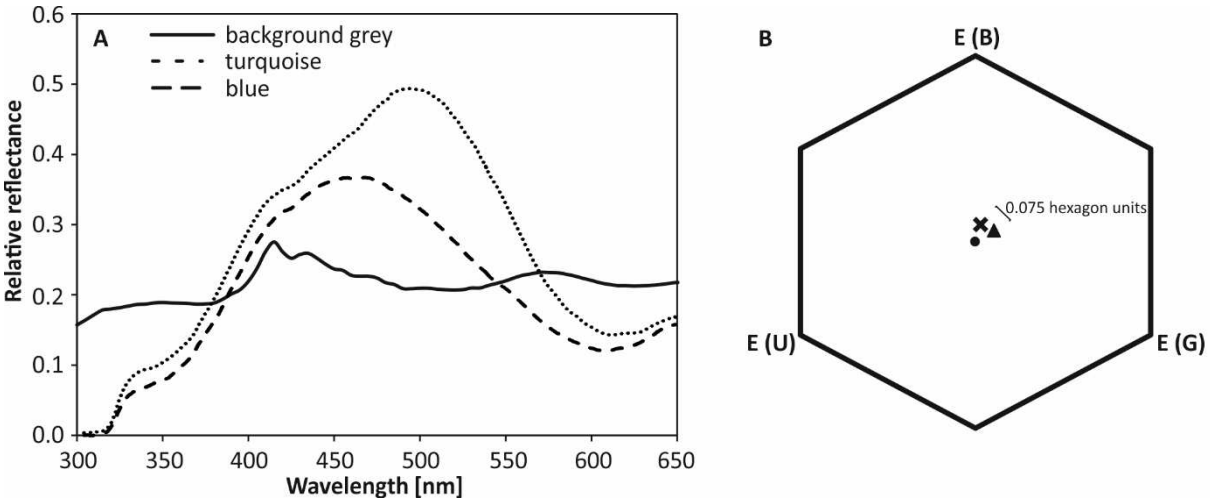

Fig 21 *Color stimuli qualities.* **A** Spectral reflectance of stimuli. **B** Loci of color stimuli in a color hexagon. The cross marks the location of the blue stimulus, while the triangle indicates the location of the turquoise stimulus. The hexagon's center is indicated by the dot. See text for details.

Individual bees were randomly assigned to one of three treatment groups: one group of bees received absolute conditioning with two rewarded target hangers (either blue or turquoise) and two unrewarded grey hangers being of the same grey as the background. I am aware that due to the experimental setup, the bees in this group have to discriminate between two types of hangers (colored vs. background-like grey) and hence are confronted with an "easy" differential conditioning task. Nevertheless, in regard to the chromatic information provided by the stimuli, we retain the terminology of absolute conditioning. This is consistent with literature about absolute visual conditioning in free-flying bees, where some form of alternative is presented to allow quantification of choices (Giurfa 2004). A second group of bees was trained using differential conditioning, i.e. these bees had to discriminate between two blue and two turquoise hangers; on one color (either blue or turquoise) sucrose solution (CS+) was provided and the other (remaining) color (CS-) was presented with pure water. A third control group (grey group) was confronted with two grey hangers providing sugar solution and two grey hangers offering pure water, thus these bees could not use any (visual) cues to discriminate the rewarded from the unrewarded stimuli, and thus no learning effect at all is expected.

Each of the 43 bees was trained for a total number of 50 decisions (approx. 15-20 foraging bouts). A decision was counted when a bee made any contact with the landing platform or solution. All groups and stimuli were tested in a pseudorandomized order.

I am aware that sensory exposure can affect microglomeruli distribution (Scholl *et al.* 2014, Muenz *et al.* 2015). However, with the recruitment procedure from a feeder dish it was ensured that all tested bees were of the same ontogenetical state (foragers) with fully maturated brains (Muenz *et al.* 2015). Moreover, due to a random assignment of individual bees to the experimental groups, potential learning-dependent changes should not be masked by age variation.

Choice Test

Following the training phase, each bee was allowed to imbibe sucrose solution on the next visited CS+ hanger until it was satiated and returned to the hive. After returning to the test site, each bee (of all three tested groups) was individually tested for its color preference in an unrewarded choice test, where two hangers with blue stimuli and two hangers with turquoise stimuli were presented on the rotating screen in a pseudorandom arrangement. The first 20

choices were counted, with a choice being scored when the bee touched or landed on a hanger.

Bee maintenance following behavioral testing

To allow for complete long-term memory formation (including protein synthesis; Wüstenberg *et al.* 1998), all bees that completed the behavioral experiments were maintained for three days in constant darkness at 27°C and 60-70% humidity. An additional group of bees was caught directly from the gravity feeder, to obtain a control group with the same ontogenic state (forager bees), but without experience of the rotating screen setup and its operant requirements, and was also put for three days into the dark. For this purpose, bees were caged individually in polystyrene tubes (6 cm in length and 2 cm diameter), closed by foam plugs and furnished with a 1 x 4 cm piece of wax panel and a feeding dish, containing a water-solved mixture of glucose and fructose.

Immunohistochemistry

After maintaining the bees for three days, synaptic complexes in the mushroom body calyces were visualized by means of immunohistochemistry using whole-mount preparations as described by Groh *et al.* (2012) and Muenz *et al.* (2015). Briefly, each bee was chilled on ice and the head capsule was opened frontally. After removal of tracheae and secretory glands, the heads were immediately transferred to 4% formaldehyde (FA) in phosphate-buffered saline (PBS), immersed overnight at 4°C and then washed in PBS (3 x 10 min). The heads were then fixed in dental wax and dissected in PBS. The isolated brains were first permeabilized in 2% Triton X-100 (Tx) in PBS for 10 min, then washed in 0.2% PBS-Tx (2 x 10 min) and eventually blocked in 2% normal goat serum (NGS) in 0.2% PBS-Tx for one hour at RT. For anti-synapsin immunohistochemistry, brains were incubated with the monoclonal primary antibody against the *Drosophila* synaptic vesicle associated protein synapsin I (SYNORF1; kindly provided by Dr. E. Buchner, University of Würzburg, Germany), diluted 1:50 in 0.2% PBS-Tx with 2% NGS for four days at 4°C. After rinsing in PBS (5 x 10 min), brains were incubated in CF488-conjugated goat anti-mouse secondary antibody (1:250) in PBS with 1% NGS for four days at 4°C. Brains were finally washed in PBS (5 x 10 min), dehydrated in an ascending ethanol series (30%, 50%, 70%, 90%, 95%, 3 x 100%, each step lasting 10 min) and cleared and mounted in methyl salicylate.

Laser scanning confocal microscopy, processing and data acquisition

Whole mount preparations were examined using a laser scanning confocal microscope (Leica TCS SP2 AOBS, Leica Microsystems AG, Wetzlar, Germany) with optical sections being taken at a resolution of 1,024 x 1,024 pixels (**Fig 22A**). For calyx reconstruction and volume measurements, optical sections at 5 µm intervals (HC PL APO objective lens: 10x/0.4 NA imm; digital zoom 3.5-4.0) were taken entirely through one of the randomly chosen medial calyces, and for analysis of MG density, high resolution scans were taken from the lip and dense collar region up to a depth of 10 µm at 0.5 µm intervals (63x/1.4 NA imm, digital zoom 2; **Fig 22 B,C**). Digital image stacks were further processed by means of 3D software (AMIRA 5.3; FEI Visualization Sciences Group, Düsseldorf, Germany). Calyces were digitally reconstructed for volumetric analysis by manually tracing the neuropil boundaries of the lip, collar (devided in dense and loose region; Groh *et al.* 2012), and basal ring on each optical section with subsequent interpolation (**Fig 22D**). The number of MG was estimated by counts of large synapsin-positive boutons analyzed in cubic volumes of 1,000 µm³ in three regions: two cubes each in the medial and lateral antennal lobe tract (m- and lALT; nomenclature after Ito *et al.* 2014) innervated lip region, and three cubes in the dense collar region (**Fig 22 B,C**; see Groh *et al.* 2012 for details). All counts were done in a blind manner without knowledge about the experimental group, and MG number was region-specific averaged per individual. To obtain estimation about the region-specific total MG number per calyx, mean MG number per 1,000 µm³ cube was multiplied by the subregion's volume.

Statistical analyses

For the behavioral experiments, the proportion of correct decisions (decision towards CS+) was calculated considering blocks of 10 trials for the three respective experimental groups, and compared to random choice level (0.5) by means of a Wilcoxon test after arcsin square root transformation. For the "grey" control group, two out of four grey stimuli were at the beginning of the experiment randomly defined as "target" and the remaining two as "distractor" to record the proportion of virtually "correct" landings. Group performance in the color choice test was compared to random choice level on the basis of the foragers' proportion of correct decisions using a Wilcoxon test after arcsin square root transformation. For the "grey" control group, the blue stimulus was declared as "correct" and the turquoise stimulus was declared as "incorrect", to obtain the proportion of "correct" landings. This group

additionally served as a test group for a potential preference towards either the blue or the turquoise stimuli. Volumes of brain regions and MG numbers were compared among experimental groups using Kruskal-Wallis-H test. Possible differences between MG number of

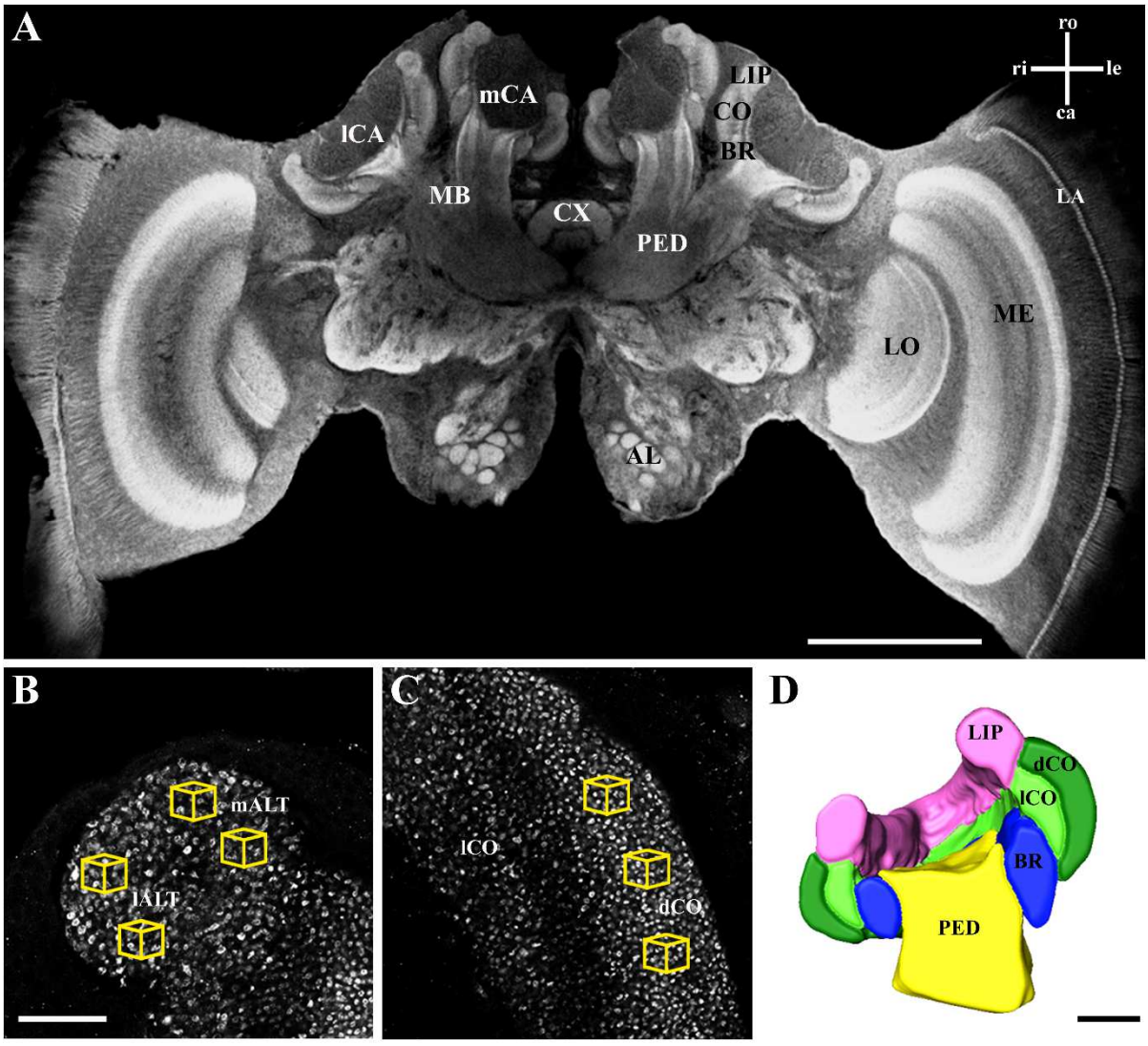

Fig 22 *Synapsin immunostaining and 3D calyx reconstruction of a forager honeybee brain*. **A** Confocal image of a frontal section through the brain after whole-mount immunolabeling for synapsin. Calyx volume and MG density were quantified in one of the medial calyces (mCA). In the magnified view of the lip (**B**) and the collar (**C**), synapsin-labeled projection neuron boutons (MG) were counted in defined volumes (1000 µm³; yellow cubes) in three regions: mALT innervated lip, lALT innervated lip, and dense collar (dCO). **D** Cross section of the volume reconstruction of the mCA rendered from confocal image stacks. AL, antennal lobe; BR, basal ring; mCA, medial calyx; lCA, lateral calyx; CX, central complex; dCO, dense collar; lCO, loose collar; LA, lamina; LO, lobula; MB, mushroom body; ME, medulla; PED, peduncle. Axes: ca, caudal; le, left; ri, right; ro, rostral. Scale bar in **A** is 500 µm, in **B** (and **C**) 25 µm, and in **D** 100 µm. Image scan from **A** is kindly provided by Kornelia Grübel

lALT and mALT region in the MB lip was calculated using Mann-Whitney-U test. Correlation between learning performance (based on the individual's number of correct landings during the 50 conditioning trials) and number of MG was calculated by means of Pearson correlation.

Results

Color conditioning and choice test

Color learning in the absolute conditioning group occurred rapidly, and bees were able to significantly choose the target color already within the first block (**Fig 23**; trial 1-10: 0.67 ± 0.04; P=0.005, Z=-2.825) with an accuracy level of 67%. The accuracy increased with the number of trials and reached a level of 88% in the last block (trial 41-50). Means of all blocks

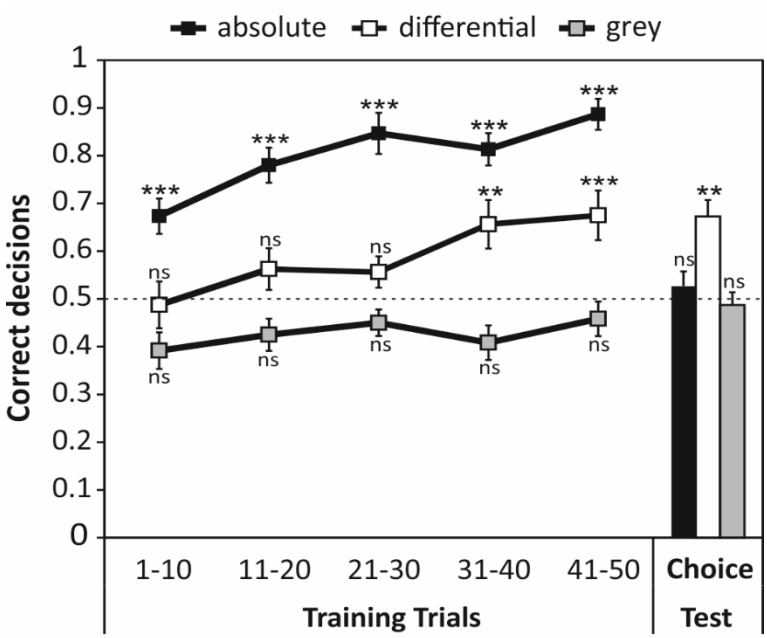

Fig 23 *Learning performance and color discrimination (choice) test of bees of three experimental groups.* One group of bees was trained with absolute conditioning to one color (against the grey background, black squares). A second group received differential conditioning with one color rewarded and a second color unrewarded (open squares). A third (control) group experienced the training without conditioning to color stimuli (grey squares). See material and methods for the definition of "correct" decision in the grey control group. All groups completed 50 conditioning trials, followed by a choice test, where all bees had to choose between the two colors used in the experiment (blue and turquoise). Horizontal grey dashed line indicates chance level (random choice). All values are mean proportion (±SEM) of correct decisions; **p<0.01; ***p<0.001; ns: not significant; absolute: N=15; differential: N=16; grey: N=12

were significantly different from chance level (trial 11-20: 0.78 ± 0.04, P<0.001, Z=-4.964; trial 21-30: 0.84 ± 0.04, P<0.001, Z=5.267; trial 31-40: 0.81 ± 0.03, P<0.001, Z=5.133; trial 41-50: 0.88 ± 0.03, P<0.001, Z=6.292). Bees of the differential conditioning group chose randomly during the first three blocks (**Fig 23**; trial 1-10: 0.48 ± 0.05, P=0.983, Z=-0.131; trial 11-20: 0.56 ± 0.04, P=0.251, Z=-0.729; trial 21-30: 0.55 ± 0.03, P=0.268, Z=-0.688) but increased the proportion of correct choices in the course of the training. From the fourth block on, bees significantly preferred the target color over the distractor color (trial 31-40: 0.65 ± 0.05, P=0.028, Z=-2.109; trial 41-50: 0.67 ± 0.05, P=0.009, Z=-2.653). Bees of the control group chose randomly among rewarded and unrewarded grey stimuli throughout the entire experiment (trial 1-10: 0.39 ± 0.04, P=0.078, Z=-1.516; trial 11-20: 0.42 ± 0.03, P=0.138, Z=-1.170; trial 21-30: 0.45 ± 0.03, P=0.175, Z=-0.972; trial 31-40: 0.40 ± 0.04, P=0.083, Z=-1.402; trial 41-50: 0.46 ± 0.04, P=0.388, Z=-0.792). When testing the bees in the subsequent color choice test for their preference for either blue or turquoise stimuli, bees of the absolute conditioning group (0.52 ± 0.03, P=0.392, Z=-0.742) and the grey control group (0.48 ± 0.03, P=0.194, Z=-0.895) chose randomly between stimuli; in contrast, bees of the differential conditioning group chose significantly more often the previously rewarded (correct) stimulus compared to the non-rewarded stimulus (0.67 ± 0.03, P=0.007, Z=-2.844).

Neuroarchitecture of the MBs

For all experimental groups, no significant differences were observed in the volume of the calyx or its substructures, the dense collar region and the lip (**Fig 24**). The mean volume per calyx differed by less than 10% among groups (absolute: 12.7 ± 0.2 x 10^6 µm³; differential: 13.0 ± 0.4 x 10^6 µm³; grey control: 12.5 ± 0.4 x 10^6 µm³; feeder control: 11.9 ± 0.7 x 10^6 µm³) with no statistical significance (P=0.899, chi²=0.590). The same was true when considering the volumes of substructures of the calyx; no significant differences were detectable among treatment groups in the lip region (P=0.872, chi²=0.705; absolute: 4.6 ± 0.2 x 10^6 µm³; differential: 4.7 ± 0.2 x 10^6 µm³; grey control: 4.7 ± 0.2 x 10^6 µm³; feeder control: 4.4 ± 0.3 x 10^6 µm³) and the dense collar region (P=0.307, chi²=3.608; absolute: 4.7 ± 0.1 x 10^6 µm³; differential: 5.1 ± 0.2 x 10^6 µm³; grey control: 4.7 ± 0.2 x 10^6 µm³; feeder control: 4.5 ± 0.3 x 10^6 µm³). For comparison of MG number among groups, data of lALT and mALT regions in the lip were pooled, as no significant differences occurred (MWU; absolute: P=0.909, Z=-0.115; differential: P=0.505, Z=-0.666; grey control: P=0.664, Z=-0.434; feeder control:

P=0.162, Z=-1.398). Between experimental groups, no significant differences were found in the number of MG per 1000 µm³ for the lip (P=0.745, chi²=1.231; absolute: 36.6 ± 2.3 MG/box; differential: 34.5 ± 1.4 MG/box; grey control: 37.3 ± 2.0 MG/box; feeder control: 36.7 ± 2.3 MG/box) or the dense collar region (P=0.266, chi²=3.955; absolute: 62.4 ± 2.8 MG/box; differential: 61.2 ± 2.5 MG/box; grey control: 68.1 ± 2.9 MG/box; feeder control: 63.5 ± 2.9 MG/box; **Fig 25A**). Estimation of total MG numbers in the lip and dense collar regions also revealed no significant differences among groups, neither for the lip (P=0.926, chi²=0.466; absolute: 15.6 ± 0.8 x 10^4 MG/calyx; differential: 15.9 ± 0.6 x 10^4 MG/calyx; grey control: 16.6 ± 0.9 x 10^4 MG/calyx; feeder control: 15.6 ± 1.0 x 10^4 MG/calyx), nor for the collar (P=0.310, chi²=3.587; absolute: 27.3 ± 1.3 x 10^4 MG/calyx; differential: 31.1 ± 1.5 x 10^4 MG/calyx; grey control: 30.0 ± 1.0 x 10^4 MG/calyx; feeder control: 27.6 ± 1.7 x 10^4 MG/calyx; **Fig 25B**).

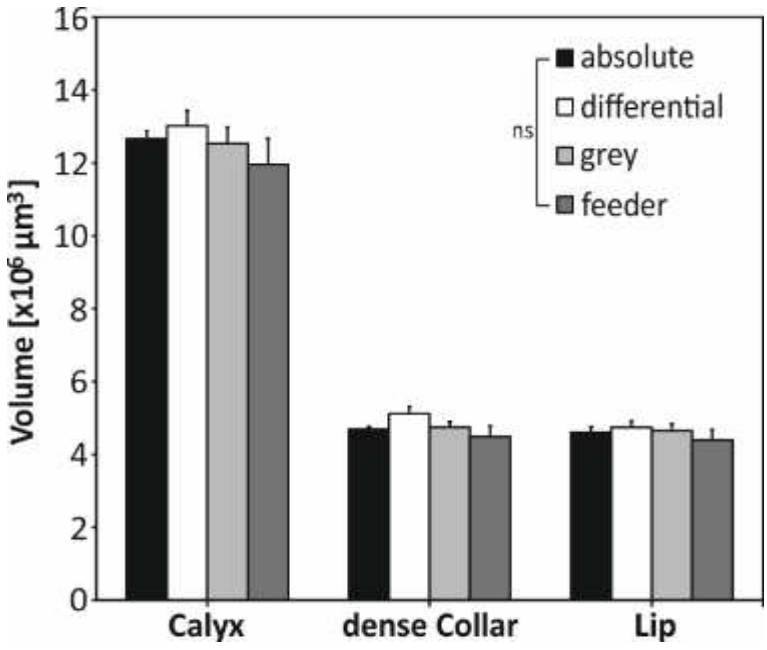

Fig 24 *Volume of the entire MB calyx and MB calyx subcompartments (dense collar and lip).* No differences were found among experimental groups for the volumes of the entire calyx, dense collar and lip regions. ns: not significant; absolute: N=13; differential: N=14; grey: N=12; feeder: N=10

Possible correlation between behavioral performance and neuroanatomy

Since recent studies have shown a correlation between MG number and olfactory learning and long-term memory in bees and ants (Hourcade *et al.* 2010, Falibene *et al.* 2015), I tested for possible correlations between MG density or number, and learning performance,

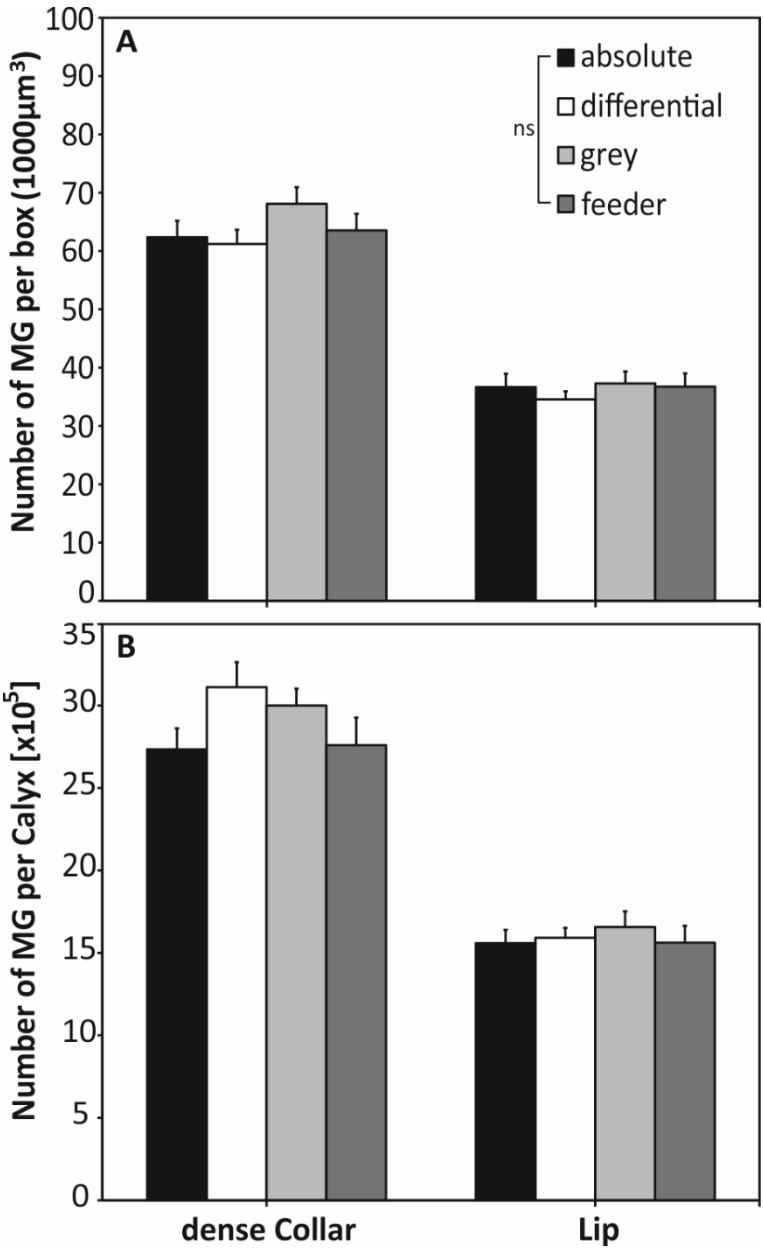

Fig 25 *Number of microglomeruli* per cube (10x10x10µm in size, **A**) and extrapolated number of MG per calyx (**B**). No differences in MG numbers were found among groups in any region (dense collar, lip), either when counted per cube or extrapolated to the total number (per calyx). ns: not significant; absolute: N=13; differential: N=13; grey: N=12; feeder: N=10

measured as total number of correct landings per individual. The number of correct landings in the absolute conditioning group significantly correlated negatively with the number of MG per 1000 µm³ box both in the lip (P=0.038, r=-0.580, **Fig 26**) and the dense collar region

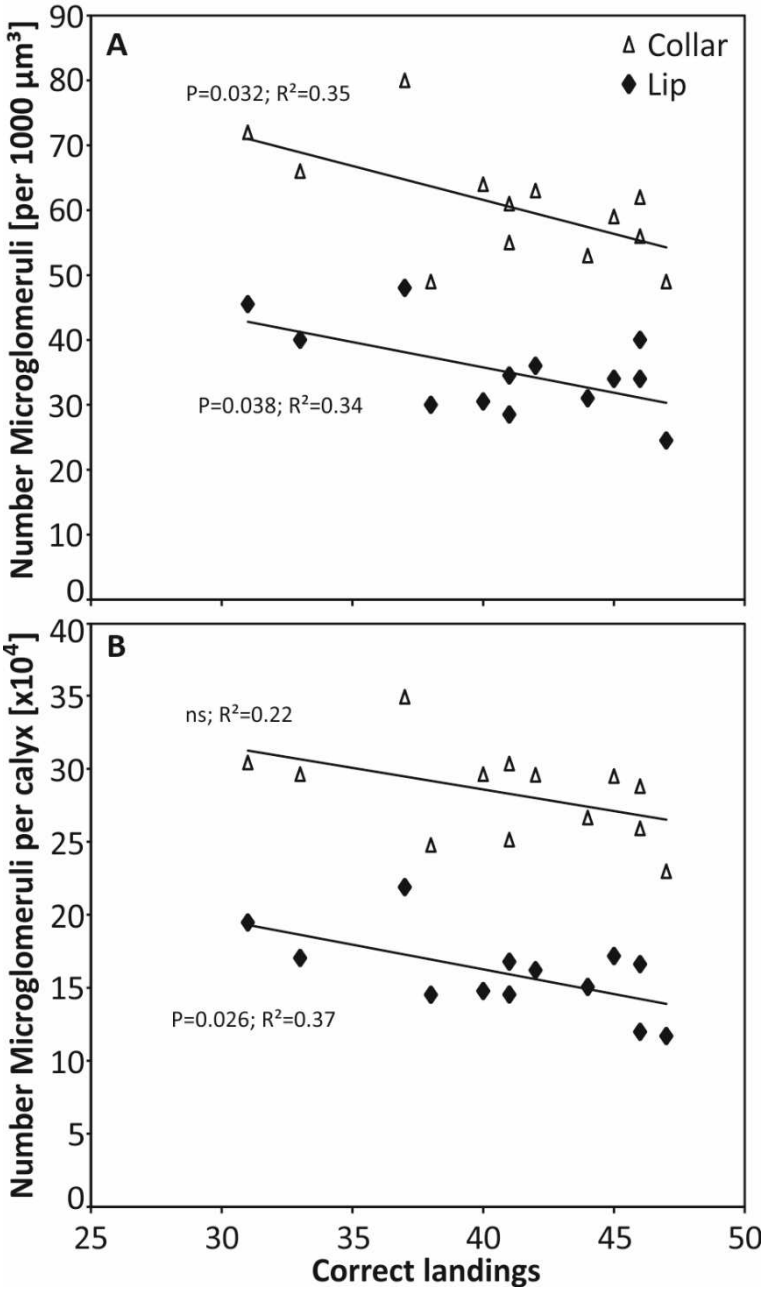

Fig 26 *Correlation between numbers of MG and numbers of correct landings in absolute conditioning experiments*. Number of correct landings significantly correlated with an individual's MG number in both calyx subregions, the lip and the dense collar. This effect was significant for the average number of MG per cube (**A**) and, for the lip, when extrapolated to whole volume (**B**). ns: not significant; N=13

(P=0.032, r=-0.594). This correlation was also significant when the number of correct landings was correlated with total number of MG in the lip (P=0.026, r=-0.612), but not in dense collar region (P=0.103, r=-0.472). In contrast, no significant correlation was found for any of the other experimental groups regarding MG density (differential: lip: P=0.520, r=-0.180, collar: P=0.912, r=-0.031; grey control: lip: P=0.325, r=-0.311, collar: P=0.853, r=0.060) and total MG numbers (differential: lip: P=0.609, r=-0.150, collar: P=0.598, r=-0.154 ; grey control: lip: P=0.275, r=-0.361, collar: P=0.660, r=-0.150).

Discussion

Honeybees are able to successfully associate color stimuli with a sugar reward (von Frisch 1914). However, learning speed and accuracy depend on the type of conditioning (Giurfa 2004, Dyer and Neumeyer 2005, Avargues-Weber and Giurfa 2014). Here it was shown that bees confronted with an easy absolute conditioning task (one rewarded color stimulus vs. unrewarded background grey) quickly learned the association. No innate preference towards either of the color stimuli was observed, as individuals of the grey control group chose randomly between the respective blue or turquoise stimuli in the final choice test (**Fig 23**). However, bees trained in the absolute conditioning task failed to discriminate the learned color from a novel, perceptually similar color stimulus (separated by a small color difference of 0.075 hexagon units) when presented with both colors simultaneously in an unrewarded test. When the same two colors were presented to the bees of the differential color conditioning task (one rewarded color vs. one unrewarded color), individuals learned the association much slower, and to a lower level of accuracy, but successfully discriminated between the colors in the subsequent choice test. The difference of discrimination capability of the same set of stimuli in both types of conditioning might be explained by the kind of information that has been learned by the bees during conditioning. Since the amount of information processing by the brain is limited, selective attention enables animals to focus on the most important or easiest accessible features of a given stimulus, rather than learning all potentially available information (Zentall and Riley 2000, Dukas 2004). Different attention mechanisms might be involved in both types of conditioning, accompanied by a stronger distraction impact of non-target stimuli but higher general attention levels in differential conditioning (Giurfa 2004, Chittka and Raine 2006, Spaethe *et al.* 2006, Avargues-Weber *et al.*

2010, Morawetz and Spaethe 2012, Avargues-Weber and Giurfa 2014), resulting in a slower acquisition curve, but very fine-tuned color discrimination.

At the neuronal level, the MBs were shown to be involved in processing selective attention-like mechanisms in visual discrimination in *Drosophila* (van Swinderen and Greenspan 2003). Furthermore, the mushroom bodies have a central function in the formation and long-term storage of associative memories (Heisenberg 1998, Menzel 2001). For olfactory information, memory storage goes along with a modification of the microglomerular organization, i.e. an increase in the connectivity of the neuronal network in the mushroom body lip region (Hourcade *et al.* 2010, for ants see Falibene *et al.* 2015). In the present study, no similar effects were found on the number of microglomeruli in the collar after visual conditioning for respective types of conditioning (**Fig 25**). It is possible, however, that a potential learning-dependent change in the number of MG after visual conditioning may be masked by the layered structure of the collar (**Fig 22C**). The MB dense collar region is innervated by neurons from different parts of the inner layers of the medulla (Paulk *et al.* 2009b, Dyer *et al.* 2011) and segregate into five strata (Gronenberg 2001, Ehmer and Gronenberg 2002). All MB input neurons in the collar are color sensitive (Paulk and Gronenberg 2008), but whether the different layers are uniformly activated during color learning is currently unknown. In contrast to visual afferent projections from the medulla, lobula projection neurons terminate besides the lip and basal ring in the light collar region, rather than the outer rim dense collar (Paulk and Gronenberg 2008). However, an accurate estimation of MG in the light collar is difficult due to its irregularity in structure and MG densities (Groh *et al.* 2012). Compared to the more homogenous conditions in the MB lip, this layered type of projection pattern causes a rather heterogeneous distribution of visually innervated MG in the collar.

A major difference to the olfaction study by (Hourcade *et al.* 2010), in which a learning-induced increase in the number of MG was found in the lip, concerns the conditioning procedure of the bees. While Hourcade *et al.* (2010) kept the bees in boxes for seven days after eclosion and maintained them in confinement for three days in constant darkness after conditioning, here free-flying forages were tested since a non-restrained condition appears essential for fine color learning in bees (Niggebrugge *et al.* 2009, Lichtenstein *et al.* 2015). Thus, the stimuli-rich natural environment, higher activity rates and motoric requirements, in our case, might have masked a possible effect of the color learning on MG density and number. With this study, a match to the natural and ecologically relevant environment was

aimed, in which free flying bees have to find, operate and memorize rewarding flowers. Interestingly, in a recent study on olfactory long-term memory and associated changes in MG organization in the MB lip of leafcutter ants, Falibene *et al.* (2015) found a significant increase of MG number two days after learning in freely moving workers of unknown age. It is therefore assumed that potentially induced subtle changes of visual MG may remain undetected by the here applied quantification technique due to the complex layered organization of MG in the honeybee collar region compared to the more homogenous lip.

The current study found a significant negative correlation between number of MG and learning performance, measured as numbers of landings on the correct target stimulus (**Fig 26**), in the absolute discrimination task. This finding suggests that individuals with a lower number of MG perform better in a color learning task. Although the functional significance is currently unclear, one can speculate an effect of experience underlying this correlation: honeybees increase their foraging performance over lifetime (experience, Dukas and Visscher 1994), whereas sensory exposure (Krofczik *et al.* 2008, Scholl *et al.* 2014) and increasing age (Muenz *et al.* 2015) correlate with a decrease in MG density and total number (as also found in two ant species Stieb *et al.* 2012, Falibene *et al.* 2015). Therefore, more experienced foragers (with lower MG density and number) may learn faster than unexperienced individuals (with initially higher MG density and number). The reason why the observed effect was found only in the absolute conditioning group is currently not clear, but might be caused by the fact that a potentially age-dependent increase in foraging performance (Dukas and Visscher 1994) and hence a higher number of correct landings, is most pronounced and experimentally observed for relatively simple discrimination tasks. Experienced foragers exhibit a higher flower constancy (Hill *et al.* 1997), while novices might operate in a more explorative manner, i.e. are more prone to visit novel and unrewarding stimuli. In contrast, performance in more sophisticated tasks, like fine color discrimination learning, might be more sensitive to general differences between learning capabilities of individual bees, which are independent of age or foraging experience but more related to personality (Scheiner *et al.* 2003, Tautz *et al.* 2003, Muller and Chittka 2012). So far, it thus remains unresolved whether neuronal correlates of visual information storage for fine color discrimination tasks, rather than the pure processing of that information, are localized in the MBs (Paulk and Gronenberg 2008). Alternatively, other central brain areas like the lateral protocerebrum (anterior optic tubercle; Mota *et al.* 2011, Mota *et al.* 2013) and the central complex (*Drosophila*: Barth and Heisenberg 1997), or even

more peripheral and upstream neuropils, like the medulla (Paulk *et al.* 2009a) and lobula (Paulk *et al.* 2008), may also play a role in visual memory depending upon the type of conditioning experienced by an individual. The latter may be supported by the observation that light exposure leads to a significant volume increase in the peripheral optic neuropils in ants (Yilmaz *et al.* 2016). Therefore, the present work underpins that the highly parallel organization of the visual system requires future studies, which aim to link color learning experiments with neuronal plasticity underlying long-term memory formation, to take more brain subdivisions and fine structure (e.g. different collar layers in the calyx) into consideration. Moreover, a potential approach is provided to combine, within subject, complex color learning type behavioral experiments with neuroanatomical analyses to investigate the visual memory trace. Alternatively, a promising approach to untangle this issue might be color learning under more controlled environmental conditions, using recently developed methods of visual conditioning of the proboscis extension response (Dobrin and Fahrbach 2012, Riveros and Gronenberg 2012, Jernigan *et al.* 2014, Lichtenstein *et al.* 2015) or pharmacological approaches to inhibit protein synthesis prior to MG quantification to prevent neuronal reorganization (see also *chapter II*). Such approaches will be of high value for understanding how these important pollinators make decisions in complex ecological conditions.

Chapter VI – Synopsis

Honeybees and bumblebees possess sophisticated cognitive abilities in order to accomplish their daily life duties. When foraging for food, they have to recognize rewarding flowers and learn their floral characteristics (odor, color, shape, etc.) as well as spatial distribution and temporal properties of the flowering plant (Proctor and Yeo 1972, Barth 1985). The information provided by the resources-offering plant is used in different ways by honeybees and bumblebees: honeybees exhibit a pronounced flower constancy and hence tend to exclusively visit certain flower species during single foraging trips (Free 1963, Hill *et al.* 1997). Bumblebees, on the other hand, are less faithful to one floral type but more focused on spatial information, which they use for traplining (Free 1970, Jakobsen *et al.* 1995, Saleh and Chittka 2007, Lihoreau *et al.* 2010). With both strategies, stimuli of different modalities, as for example visual and olfactory, must be associatively learned and stored as memories for later retrieval. Stimulus processing of visual and olfactory information in the brain has been investigated and untangled to great extents in honeybees (particularly olfaction; reviewed by Galizia and Rössler 2010, Sandoz 2012) and bumblebees (basically vision; Dyer *et al.* 2011, Avarguès-Weber *et al.* 2012) and characteristics are assumed to be common for both closely related bee species (Dyer *et al.* 2011). In contrast, the memory traces are still enigmatic and knowledge particularly for the storage of visual content is scarce. However, for questioning the visual memory trace, it might be worth to first look at the processes facilitating olfactory memory storage, where large progress was achieved in honeybees in the last decades (reviewed by Menzel 2001, Menzel 2013).

Scent perception occurs with olfactory receptors located on the antennae, and sensory neurons expressing the same receptor protein converge on glomeruli in the antennal lobe (AL). The odor is specifically encoded by the combinatorial activity pattern of 160 glomeruli in worker bees (Sachse *et al.* 1999). Information is processed by local interneurons within the AL and transmitted by projection neurons (PN) to the mushroom body (MB) and lateral horn (LH) via a dual tract (mALT and lALT; Brill *et al.* 2013). In the MB, the neural excitation diverges, as approx. 800 PN synapse onto more than 100,000 MB intrinsic neurons, the Kenyon cells (KC). The neuronal circuits, which connect PNs to KCs, are organized in distinct microglomeruli (MG), each comprising a single presynaptic PN bouton, as well as approximately 6-12

postsynaptic dendritic spines of KCs and inhibitory recurrent neurons (Ganeshina and Menzel 2001, Rössler and Groh 2012). Single KCs either collect excitations from one of the calyx' subcompartments (olfactory lip, visual collar, multimodal basal ring) or across them, and project to the MB output lobes (α and β lobe). The KCs converge to approx. 400 extrinsic neurons, which project to various brain parts: e.g. to the lateral horn, LH, or back to the calyx as inhibitory recurrent neurons. Memory formation, however, requires a link between the stimulus processing pathway and the information about its meaning (e.g. appetitive, aversive). Such reinforcing mechanisms are facilitated by octopaminergic and dopaminergic neurons, like the honeybee's octopaminergic VUMmx1 neuron. It receives its input, e.g. in response to sucrose stimulation, from the subesophageal ganglion and merges with the olfactory pathway at three sites: the antennal lobes (AL), the LH, and within the calices' lip region (Hammer 1993). These sites of information convergence have been suggested as potential locations for the olfactory memory trace. In AL, manipulation of the octopamin availability by injection or receptor blocking, as well as local cooling and cAMP uncaging (for the role of cAMP in learning see *chapter I*) affected memory formation (transition from STM to LTM; reviewed in Menzel 2013). Moreover, the activity patterns of glomeruli possess a certain plasticity in the course of associative odor learning and the neural representation of learned odors change in the AL with experience (Faber *et al.* 1999). The second potential site of olfactory memory storage, is expected within the MB. PN ascending from AL converge with KC in association with VUMmx1 and GABAergic neurons in the lip of the calyces in discrete MG. The density of MG is subjected to dynamic changes as a function of the bee's age and experience, and increases in the lip during the consolidation of olfactory memory (Hourcade *et al.* 2010). The high number of intrinsic neuronal cells, the convergence of multimodal information and the matrix-like plastic organization of the calyx provide strong evidence for the assumption that the memory trace lies within the MB.

The advanced knowledge in olfactory memory formation is based on the availability of an approach which allows for behavioral experiments under controlled conditions and simultaneous access to the bee brain: the proboscis extension response (PER) assay (Kuwabara 1957, Bitterman *et al.* 1983, Giurfa and Sandoz 2012, Matsumoto *et al.* 2012). While this approach has long been applied for olfactory conditioning experiments, it was only recently successfully adapted for visual experiments in honeybees (Dobrin and Fahrbach 2012,

Jernigan *et al.* 2014) and bumblebees (Riveros and Gronenberg 2012, Lichtenstein *et al.* 2015). This progress also paved the way for the present dissertation research.

As mentioned above, the initial aim of this dissertation research was to get access to the visual memory trace that is beyond the mere stimulus processing (the visual processing pathway is described in *chapter I*, **Fig 5**). For this purpose, two different potential approaches were targeted: first, establishing the use of immediate early genes (IEG) as genetic markers and monitoring tools for neuronal activation of brain domains involved in visual associative learning and memory formation. With this first approach, early processes underlying formation of long-lasting memories were addressed. The second approach focused on the analysis of late consequences of memory formation, including the reorganization of the synaptic network within the visual domains of the MB calyces.

Immediate early gene expression

For the application of IEGs as mapping tools for neuronal activation in the context of memory formation, three requirements have to be met: first, the formation of visual long-term memories (LTM) must generally depend on the transcription of genes, putatively comprising IEGs and their potential target genes. Second, stimuli that induce the expression of potential IEGs must be defined. And third, a link between the expression of candidate IEGs and visual learning and memory formation must be validated. These prerequisites were successively elaborated in the following.

To validate that the formation of visual LTM generally requires gene transcription, bumblebees were trained in an absolute conditioning task to a monochromatic light stimulus and later injected with the transcription inhibitor actinomycin D *(chapter II)*. In comparison to control bees that either remained untreated or were injected with the solvent (PBS) alone, it was shown that gene transcription is indeed necessary for the formation of a visual LTM (tested after 3 days), but not for MTM (tested after 6 hours). Considering the timing of ActD injection (3 hours following conditioning), supposedly the expression of late response genes (LRG) is inhibited. These LRGs are regulated by transcription factors encoded by IEGs and include genes whose products are involved in modifications of synaptic transmission and neuro-structural reorganization (Tischmeyer and Grimm 1999, Clayton 2000). The presumed first wave of transcription, which represents the activation of the IEGs itself, was not affected.

Thus, whether the formation of a visual MTM is, as opposed to LTM, completely independent of transcription, remains unresolved. Data from olfactory learning, however, suggest prolonged activities of kinases but not gene transcription as the molecular substrate for the formation of MTM (Müller 2002). Moreover, a direct proof of a first transcriptional wave, as well as the precise timing and spatial distribution of the two putative transcription waves, is pending.

The mentioned first wave of transcription was addressed when efforts were made to characterize two potential IEGs in honeybees, their triggering stimuli and the spatial and temporal expression patterns in response to sensory exposure and associative learning (*chapters III* and *IV*). Both IEGs, *Amjra* and *Amegr*, encode for inducible transcription factors of the bZIP and zinc-finger type, respectively, whose orthologs in vertebrates are involved in neuronal activation underlying memory formation. With the present work it has now been shown in honeybees that both IEGs vary in their transcriptional responses to stimulation with either a (visual) light pulse or (olfactory) alarm pheromone. While *Amjra* is induced by both stimuli in all analyzed brain parts (AL, OL, MB), the X1 isoform of *Amegr* exhibits no significant expression response. Thus, *Amjra* might be considered as a honeybee IEG that is expressed within the first transcriptional wave after stimulation (approx. 30 min following stimulus onset) and is potentially suitable to monitor neuronal activation in the course of arousal (due to unspecific stimulus induction). Whether the activation in the brain is homogenously distributed or particularly pronounced in distinct sub-regions of the analyzed neuropils (AL, OL, MB), needs to be evaluated by the application of analysis methods with higher spatial resolution, as for example *in situ* hybridization. Additionally, so far it is unclear whether the upregulation of *Amjra* mRNA also results in elevated levels of the corresponding functionally active protein product. Hence, the transcription factor AmJRA and its temporal and spatial distribution by antibody-based Western blot analysis and immunohistochemistry must be addressed in future studies.

Even though *Amegr* mRNA levels were not affected by mere sensory exposure, a putative role of the gene in (visual) associative learning and memory formation is possible. This is based on the known functions of members of the orthologous family of EGR transcription factors in the nervous system of vertebrates (O'Donovan *et al.* 1999, Knapska and Kaczmarek 2004). In the present study, the presence of AmEGR protein in the honeybee brain was validated by means

of immunofluorescence staining using custom-made antibodies (*chapter IV*). Here, AmEGR was shown to be expressed in virtually all cell nuclei of the honeybee brain with a particular pronounced immune-reactivity in the Kenyon cells of the MB calyces, the putative site for memory storage. However, if *Amegr* expression is indeed learning-dependent, induction of its mRNA might be based not only on the presentation of a relevant stimulus, but also on information about its meaning (appetitive or aversive reinforcement). While presentation of a single signal has been shown to induce the putative "arousal marker" *Amjra*, some sort of associative information might be needed for *Amegr* expression. To address this issue, honeybees were trained in visual conditioning of the proboscis extension response (PER) assay and subsequently dissected at different intervals (30, 90 and 240 min) after conditioning. In the experimental group, no induction of the expression of one of the three *Amegr* isoforms was observed. Since other types of stimulation, e.g. sucrose feeding and performance of orientation flights, have been previously reported to activate the expression of *Amegr* in an IEG-typical temporal manner (peak levels at 30-60 min after stimulation; Lutz and Robinson 2013, Ugajin *et al.* 2013), the final assessment about the gene's suitability for mapping neuronal activation is pending, but might be highly specific to the type of stimulation.

To further develop the use of IEGs as neuronal activity markers in bees, additional candidate genes must be considered. Besides the two IEG that were investigated in the present dissertation research, only two more candidate genes were already tested in honeybees so far: Fos (Fonta *et al.* 1995), which is a dimerization partner of AmJra to form Ap-1, and the non-coding RNA *kakusei* (Kiya *et al.* 2007, Kiya *et al.* 2008). Potential alternatives to the transcription factors AmJra and AmEgr are IEGs encoding direct effector proteins, like Ubiquitin hydroxylase or the activity-regulated cytoskeleton-associated (Arc) protein. Both candidates have been well characterized in vertebrates and homologous representatives were identified in insects (Moguilevsky *et al.* 1994, Mattaliano *et al.* 2007). Particularly Arc, which has been shown in vertebrates to be involved in synaptic trafficking via endosomes and processes of neuronal plasticity, like long-term potentiation and long-term depression (Arc regulation and functions are reviewed in Bramham *et al.* 2008), might be a promising candidate. The mRNA of Arc is rapidly transported to dendritic sites and cellular stimulation results in a locally restricted synaptic translation (Seteward *et al.* 1998). Thus, monitoring the

Arc protein expression may give precisely defined spatial information about neuro-active brain sites.

Dynamics of MB calyx microglomeruli

The general sequence of cell-physiological events leading from STM to LTM is commonly accepted across species and comprises prolonged synaptic transmission, intracellular signaling cascades, gene transcription and translation. Eventually, long-term storage of memories is likely to be based on a structural reorganization of the neuronal network (Bailey *et al.* 1996, Sara 2000, Kandel 2001, Menzel 2001). The restructuring includes both changes in pre-existing synapses, at this modifying the strength of the synaptic transmission, and in the number of synaptic connections, facilitated by pruning and neoformation processes (Bailey and Kandel 1993, Segal *et al.* 2000). This dynamic cellular network can be considered as the structural substrate for long-lasting memories, which might be centrally stored or decentralized distributed throughout different brain areas (Thompson 1986). In insects, the mushroom bodies (MB) have been proposed as pivotal brain structures for sensory integration and associative learning, and putatively for memory storage (Heisenberg 1998, Strausfeld *et al.* 1998, Fahrbach 2006, Devaud *et al.* 2015). In the context of olfactory associative learning, coherencies between LTM formation and reorganization of synaptic circuits, so called microglomeruli (MG), within the olfactory-innervated subdomain of the MB calyx (the lip) have been found in Diptera (*Drosophila*: Kremer *et al.* 2010), as well as Hymenoptera (honeybee: Hourcade *et al.* 2010, *Acromyrmex*: Falibene *et al.* 2015). A contribution of the MB to associative and non-associative visual processing so far has only been shown in flies (Barth and Heisenberg 1997, Liu *et al.* 1999, Vogt *et al.* 2014). Thus, another aim of this doctoral thesis was to determine a potential memory trace of visual content within the MB, with particular focus on the visual information-processing collar domain of the MB calyx. Identifying the spatial traits of visual memory storage may help to further elaborate on the neuronal mechanisms behind behavioral plasticity in bee color discrimination learning (as introduced in *chapter V*).

The bee brain is suggested to possess different processing systems mediating coarse and fine color discrimination learning (Dyer *et al.* 2011). Both processes differ substantially in acquisition speed and accuracy of the memory (Dyer and Chittka 2004a, Giurfa 2004).

However, with the present work (*chapter V*), no evidence for a visual memory trace – measured as variations in the density of micoglomeruli – was found in the MB collar. Among treatment groups (absolute and differential conditioning group, as well as naïve control groups regarding color-stimulus and test apparatus), no significant learning-dependent effects on the MB neuroarchitecture were found. Since the study was conducted with free-flying honeybee foragers, potential learning-dependent slight changes in synaptic complexes might be masked by uncontrollable environmental effects, e.g. previous individual experience. Moreover, so far one can only speculate about the timing of potential events of synaptic plasticity in the course of visual memory formation. While late phases of LTM, based on structural reorganization of neuronal networks, are generally considered to be consolidated after >3 days (Menzel 1999, Menzel 2001, Menzel 2013), Falibene *et al.* (2015) reported a dynamic regulation of the number of microglomeruli in a transient manner in leaf-cutting ants' avoidance learning. To analyze the temporal coherencies in microglomeruli plasticity in honeybees and bumblebees, visual PER conditioning under standard laboratory conditions should be conducted, followed by neuroanatomical quantification of microglomeruli at different time points.

The collar possesses a layered organization and consists of several distinct strata innervated by afferent projections, which derive from different peripheral brain areas (e.g. dorsal and ventral sites of the ipsilateral and contralateral medulla and lobula; Ehmer and Gronenberg 2002, Paulk and Gronenberg 2008). This architecture depicts another challenge in monitoring synaptic plasticity in this brain area. So far, it remains largely unclear whether functional differences in information processing among the strata exist. Alternatively to the putative centrally located processing site, visual memory might be stored decentralized in various brain regions (Dyer *et al.* 2011, Avarguès-Weber *et al.* 2012) and hence a pronounced learning-dependent synaptic reorganization in a single brain domain is absent. Potential sites that have been shown to be involved in different aspects of visual processing are located in the median (central complex, reviewed in Homberg 2008, Pfeiffer and Homberg 2014) and lateral protocerebrum (e.g. the anterior optic tubercle; Mota *et al.* 2011, Mota *et al.* 2013). Also, advanced information processing and potentially some sort of storage might occur already at the visual periphery in the optic lobes of the bee brain. Lamina neurons exhibit little response variation across different wavelengths and weak horizontal processing (Menzel 1974, Ribi

1975). In contrast, medulla and lobula neuropils show a pronounced spatio-temporal segregation of the visual information (e.g. chromatic properties and motion) and possess massive horizontal processing (e.g. via broadband, narrowband, color-opponent and spatial opponent neurons interconnected by horizontal fibers; Kien and Menzel 1977a, Kien and Menzel 1977b, Hertel 1980, Hertel and Maronde 1987). It remains elusive whether the optic lobes contribute to visual memory formation, comparable to the plasticity observed in antennal lobe processing in olfactory memory formation (Grünbaum and Müller 1998, Rath *et al.* 2011), e.g. via feedback mechanisms from the MB. Promising approaches with high spatial resolution to uncover active brain sites comprise electro- and optophysiological recordings of visual circuit activity (Paulk *et al.* 2008, Mota *et al.* 2011), intracellular staining (Ehmer and Gronenberg 2002) and immunohistochemistry on appropriate IEGs. However, these techniques are not suitable to monitor reorganization of the neuronal network, which is considered as the substrate for long-lasting memories. Thus, either analyses of spatially more restricted brain domains, as for example different MB collar layers, or large-scale computation of cranial cell interactions might be needed to solve the puzzle of visual memory formation.

The present dissertation revealed similarities and putative differences of the visual system as compared to the olfactory system in bees. For both modalities, post-conditioning gene transcription is necessary for the formation of LTM, but not MTM. In contrast, the transient increase in the density of MB calyx microglomeruli found in olfactory LTM formation, could not be validated for visual LTM. Additionally, fundamental work on the potential use of IEG as markers for neuronal activation is provided to possibly pave the way to unravel the visual memory trace by use of molecular genetics.

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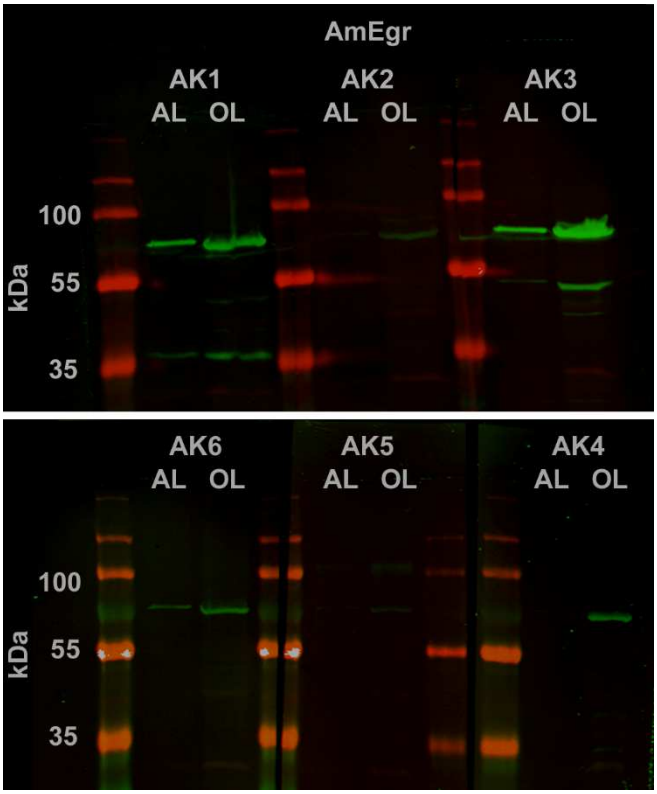
Appendix I – Supplementary Results

Test of 6 candidates of AmEgr immune-reactive antibodies

Western blot

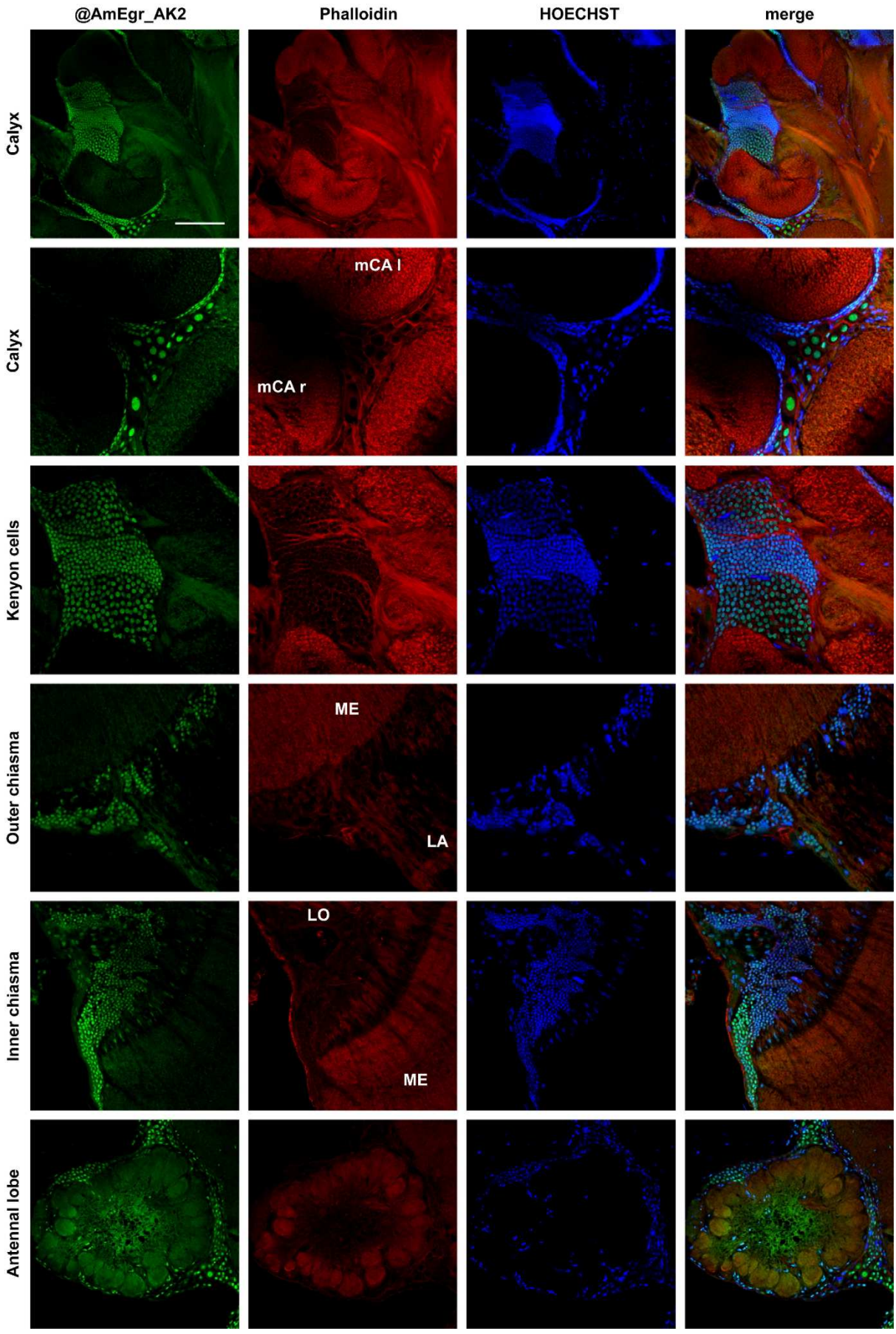
Immune-reactivity of all candidate antibodies was evaluated by Western blot analysis. The molecular weights of the AmEgr isoforms are 90, 87 and 65 kDa. AL, antennal lobe; OL, optic lobe

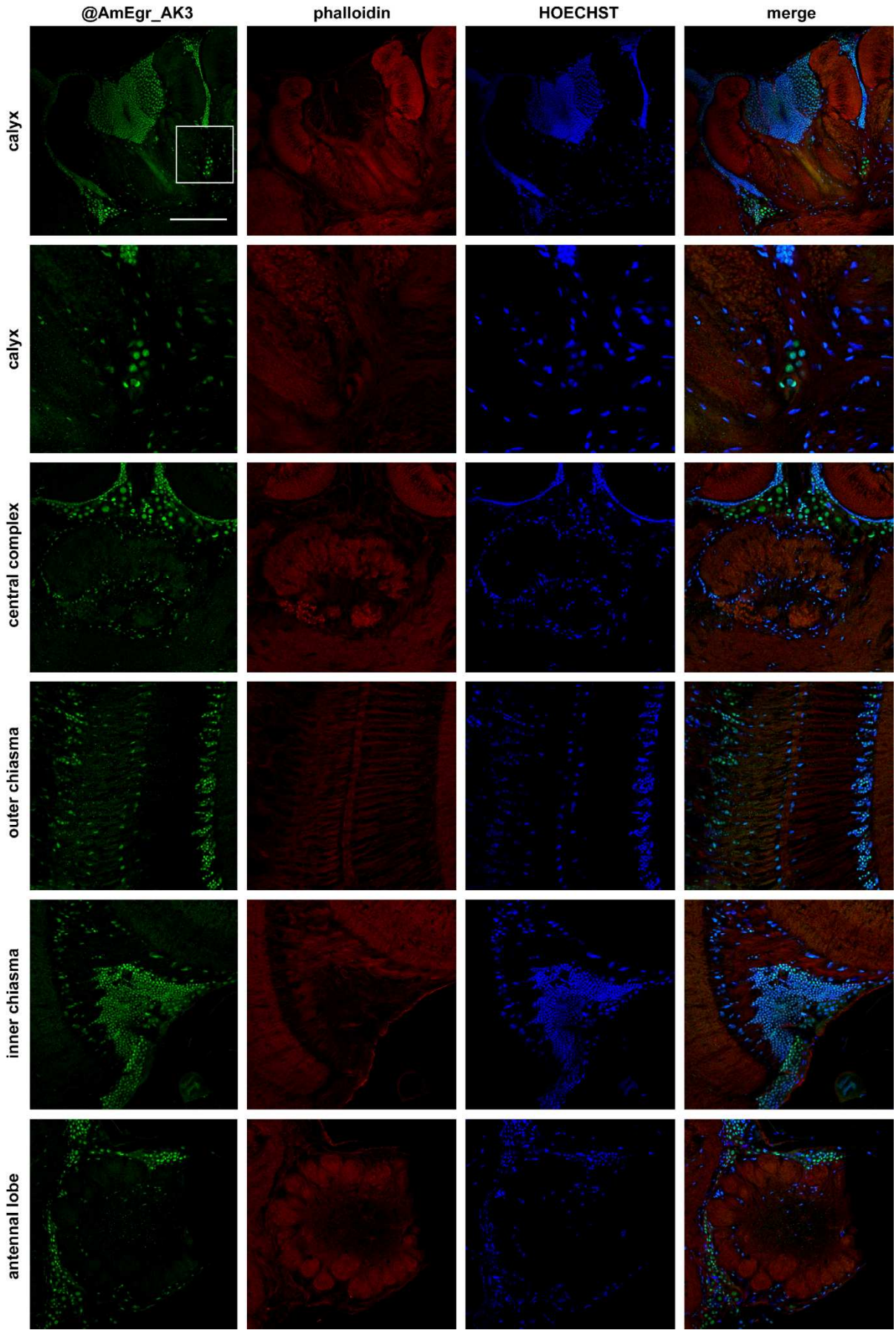


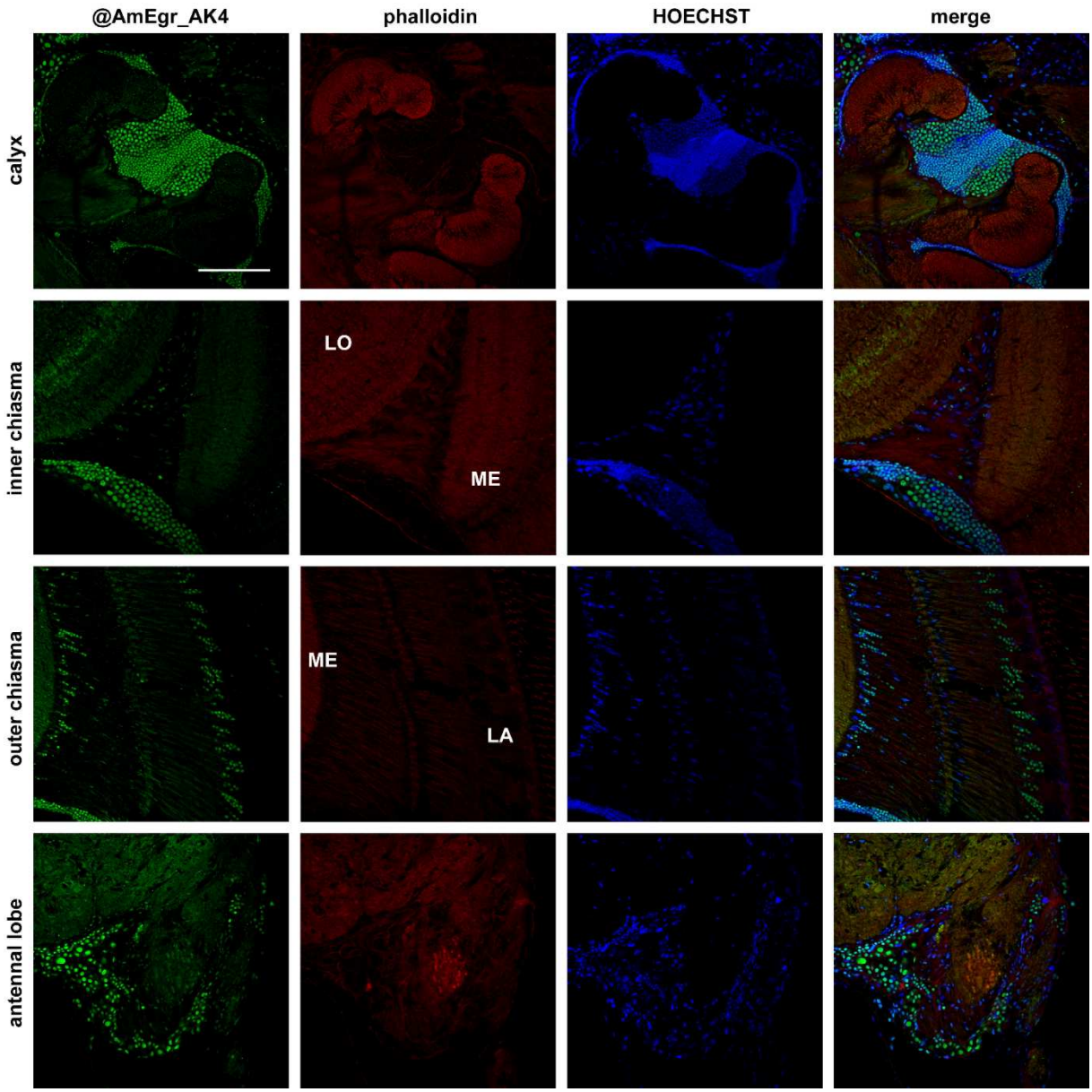
Immunohistofluorescence imaging

On the following pages, the immune-reactivity of the tested alternative anti-AmEgr antibodies is shown on frontal brain sections of worker honeybees. While no (specific) immuno-signal was observed for AmEgr_AK1, AmEgr_AK5 and AmEgr_AK6 (the latter two were designed to detect exclusively the AmEgrX1 isoform), a distinct immune-reactivity was observed for AmEgr_AK2, AmEgr_AK3 and AmEgr_AK4.

Scale bars in all upper panels of calyx images represent 100 µm, which also counts for antennal lobe images. The same scale bars indicate 50 µm for the lower panel of calyx images (for AmEgr_AK2) as well as for images of Kenyon cells and the outer and inner optic chiasma; 75 µm for central complex and 30 µm for magnified images of calyx region (both AmEgr_AK3). mCA r/l, median calyx on right/left brain hemisphere; ME, medulla, LA, lamina; LO, lobula


@AmEgr_AK2
Phalloidin
HOECHST
merge
Calyx
Calyx
mCA l
mCA r
Kenyon cells
Outer chiasma
ME
LA
Inner chiasma
LO
ME
Antennal lobe


@AmEgr_AK3
phalloidin
HOECHST
merge
calyx
calyx
central complex
outer chiasma
inner chiasma
antennal lobe


@AmEgr_AK4
phalloidin
HOECHST
merge
calyx
inner chiasma
outer chiasma
antennal lobe
LO
ME
ME
LA

Appendix II – Supplementary Material and Methods

Software

Adobe Photoshop V 7.0

CorelDraw X7

Endnote X7.5 Thomson Reuters

IBM SPSS Statistics 20

Mastercycler ep realplex software version 2.2, Eppendorf

Microsoft Office Professional Plus 2010

Microsoft Office Professional Plus 2013

Materials and devices

Acrylamide, Rotiphorese®Gel40	Carl Roth, Karlsruhe, Germany
adhesive Masterclear real-time PCR film	Eppendorf, Hamburg, Germany
6-Aminocaproic acid	Merck, Darmstadt, Germany
Balance Universal U4800p	Sartorius, Göttingen, Germany
BioPhotometer plus	Eppendorf, Hamburg, Germany
Bromphenol blue	Carl Roth, Karlsruhe, Germany
Centrifuge 5424R	Eppendorf, Hamburg, Germany
Centrifuge 5430	Eppendorf, Hamburg, Germany
Cooled incubator MIR-154	Panasonic Biomed, Etten, Netherlands
Gel documentation/Intas science imaging	Intas Sc. Imaging, Göttingen, Germany
Gel separation system	Owl Scientific, San Francisco, CA, USA
Glycerin	Carl Roth, Karlsruhe, Germany
Glycin	Ajinomoto, Tokyo, Japan

KL 1500 electronic	Leica, Wetzlar, Germany
Kombischüttler KL-2	Edmund Bühler, Hechingen, Germany
Leica MZ6	Leica Microsystems, Wetzlar, Germany
Leica VT1000 S microtome	Leica Microsystems, Wetzlar, Germany
Magnetic stirrer IKA Combimag RCH	IKA Werke, Staufen i. Breisgau, Germany
Mastercycler gradient S realplex² Mastercycler ep realplex software version 2.2	Eppendorf, Hamburg, Germany
Mastercycler gradient	Eppendorf, Hamburg, Germany
µCuvette G1.0	Eppendorf, Hamburg, Germany
2-Mercaptoethanol	Carl Roth, Karlsruhe, Germany
Methanol	AppliChem, Darmstadt, Germany
Milk powder, blotting grade	Carl Roth, Karlsruhe, Germany
Mini-Protean Electrophoresis	Bio-Rad, Hercules, CA, USA
miniSpin centrifuge	Eppendorf, Hamburg, Germany
pH meter PB-11	Sartorius, Göttingen, Germany
Polyvinylidene fluoride/PVDF membrane	Merck, Darmstadt, Germany
Ponceau S solution	Sigma Aldrich, St. Louis, Missouri, USA
Power supplier, peqPOWER 300V	VWR-Peqlab, Radnor, Pennsylvania, USA
Semi-Dry Electrotransfer Unit, Western blot	VWR-Peqlab, Radnor, Pennsylvania, USA
Sodiumdodecylsulfate, SDS	Carl Roth, Karlsruhe, Germany
Tetramethylethylenediamine (TEMED)	Carl Roth, Karlsruhe, Germany
Thermomixer comfort	Eppendorf, Hamburg, Germany
Tissuelyser LT	Qiagen, Venlo, The Netherlands
Tris	Carl Roth, Karlsruhe, Germany
twin.tec PCR 96 well plates	Eppendorf, Hamburg, Germany
Vortex-Genie 2	Scientific Industries, Bohemia, NY, USA

Antibodies

				Dilution	
Name	**Antigen**	**Donor**	**Manufacturer**	**Delivered** [µg/ml]	**Applied** **IF** **WB**
@AmEgr _AK1	AmEgr-His aa 583-592	Rabbit	Immunoglobe	60	1:1000 1 µg/ml
@AmEgr _AK2	AmEgr-His aa 583-592	Rabbit	Immunoglobe	120	1:1000 1 µg/ml
@AmEgr _AK3 ***	AmEgr-His aa 609-621	Rabbit	Immunoglobe	130	1:1000 1 µg/ml
@AmEgr _AK4	AmEgr-His aa 609-621	Rabbit	Immunoglobe	170	1:1000 1 µg/ml
@AmEgr _AK5	AmEgrX1-His aa 375-385	Rabbit	Immunoglobe	38	1:1000 1 µg/ml
@AmEgr _AK6	AmEgrX1-His aa 375-385	Rabbit	Immunoglobe	172	1:1000 1 µg/ml
IRDye680	against Rabbit	Donkey	LI-COR		1:20000
Alexa Fluor488	against Rabbit	Goat	ThermoFisher		1:250

*** @AmEgr_AK3 was applied in WB and IF experiments presented in chapter IV

Protocols

SDS-PAGE (Sodium dodecyl sulfate; polyacrylamide gel electrophoresis)

Solutions

Separation gel buffer	1.5 M Tris, pH 8.8, 0.8% (w/v) SDS
Stacking gel buffer	0.5 M Tris, pH 6.8, 0.8% (w/v) SDS
10x chamber buffer	250 mM Tris, pH 8.3, 1.9 M Glycin, 1% (w/v) SDS

Gel preparation (for two gels)

10% acrylamide separation gel:

Acrylamide (Rotiphorese®Gel 40; 40% acrylamide/bisacrylamide 37, 5:1)	3.0 ml
Separation gel buffer	3.0 ml
H_2O	6.0 ml
Temed (N,N,N';N'-Tetramethylethylendiamin)	12 µl
APS (10% (w/v) ammonium persulfate)	70 µl

6% acrylamide stacking gel:

Acrylamide (Rotiphorese®Gel 40; 40% acrylamide/bisacrylamide 37, 5:1)	450 µl
Separation gel buffer	1.0 ml
H_2O	2.55 ml
Temed (N,N,N';N'-Tetramethylethylendiamin)	4 µl
APS (10% (w/v) ammonium persulfate)	30 µl

Electrophorese with 1x chamber buffer

pre-run at 80 V, 60 mA, 40 W for 15 min
run at 120 V, 60 mA, 40 W for approx. 60-90 min

<u>Western blotting</u>

Solutions

Laemmli buffer	125 mM Tris, 6% glycerin, 2% SDS, 10% mercaptoethanol, 0.004% bromphenol blue, pH 6.8
Anode buffer I	300 mM Tris-base, 20% (v/v) methanol, pH 10.4
Anode buffer II	25 mM Tris-base, 20% (v/v) methanol, pH 10.4
Cathode buffer	40 mM 6-Aminohexanoic acid, 20% (v/v) methanol, pH 7.6

Equilibration of polyvinylidene fluoride (PVDF) membrane for 5 min in methanol and assembling of Western blot:

bottom:	6 filter papers soaked with anode buffer I
	3 filter papers soaked with anode buffer II
	PVDF membrane (equilibrated)
	Polyacrylamide Gel
top:	9 filter papers soaked with cathode buffer

Protein transfer occurs for 90 min at 54 mA per gel (20 V, 54 mA, 4 W).

For evaluation of protein transfer, the PFDF membrane was allowed to incubation for 10 min in Ponceau S red solution, followed by H_2O destaining.

Western blot immunostaining

Solutions / Materials

Blocking buffer	5% Milk powder in TBST
Protein ladder	PageRuler™ /Plus, Thermo Fisher Scientific, Waltham, Massachusetts, USA

Phenol-chloroform extraction of total RNA from frozen honeybee brain tissues

Frozen tissues are homogenized in 500 µl Isol-RNA Lysis Reagent (5Prime, Düsseldorf, Germany) by means of steel beads and a TissueLyser (Qiagen, Venlo, Netherlands) for 3 min at 40 Hz. Following a 5 minutes resting phase at room temperature (RT), 0.1 ml chloroform is added to each sample and the mixture is thoroughly shaken for 15 sec. After a second resting phase (3 min at RT), samples are centrifuged for 15 min at 12,000 x g and 4°C. The RNA-containing aqueous top phase is then transferred to a new 1.5 ml tube (Eppendorf AG, Hamburg, Germany) and mixed with 30 µl 3M NaAc, 2 µl glycogen (Thermo Scientific, Waltham, Massachusetts, USA) and 300 µl isopropanol. Following incubation for 30 min at -70°C, the samples are centrifuged for 15 min at 12,000 x g and 4°C, the supernatant is removed and the RNA pellets are washed twice with 1 ml 75% EtOH, each washing step followed by centrifugation at 7,500 x g and 4°C for 5 min. The pellets are then air dried for 10 min, resuspended in 30 µl RNase-free water (Roth, Karlsruhe, Germany) and stored at -70°C upon usage with e.g. quantitative real-time polymerase-chain-reaction (qPCR).

PCR (Polymerase chain reaction) for primer validation

Solutions / Materials

DNA polymerase	peqGOLD Hot Start-Mix S; VWR Intl., Radnor, Pennsylvania, USA
Primer	custom-made oligonucleotides; metabion Intl. AG, Steinkirchen, Germany

Reaction mix

12.5 µl	Taq DNA polymerase mix incl. dNTPs, Tris buffer, $MgCl_2$ and KCl
6.5 µl	H_2O
2.0 µl	forward primer (10 µM)
2.0 µl	reverse primer (10 µM)
2.0 µl	template cDNA

Temperature Cycling

Initial denaturation	5 min	95 °C
35 amplification cycles	1 min	95 °C
	45 sec	63 °C
	1 min	72 °C
Final elongation	5 min	72 °C
Hold		4°C

<u>Agarose gel electrophoresis</u>

Solutions / Materials

Agarose	Roti®garose NEEO Ultra-Qualität, Carl Roth, Karlsruhe, Germany
Nucleic acid stain	Midori Green Direct, Nippon Genetics Europe, Düren, Germany
DNA ladder	GeneRuler 100 bp DNA Ladder/Plus, Thermo Fisher Scientific, Waltham, Massachusetts, USA
Tris-borat EDTA	TBE: 90 mM TRIS base, 90 mM boracic acid, 2 mM EDTA-NA_2

Depending on the size of nucleic acids to be separated, 0.8-2% agarose (in TBE) gels are placed in an electrophoresis chamber and loaded with a mixture of 4-14 µl of DNA or RNA sample and 0.5 µl Midori Green. Separation occurs at 130 V for 30 min.

Appendix III – Personal information

Affidavit

I hereby confirm that my thesis entitled “Mechanisms of visual memory formation in bees: About immediate early genes and synaptic plasticity” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.
Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg,

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation “Mechanismen der visuellen Gedächtnisbildung bei Bienen: Über unmittelbar früh exprimierte Gene und synaptische Plastizität” eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.
Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg,

Ort, Datum Unterschrift

Chapter III and chapter IV of this thesis were largely adapted for two manuscripts intended for publication in peer-reviewed journals.

Statement of author contributions

Manuscript I (chapter III): Sommerlandt FMJ, Rössler W, Spaethe J. (under review). Impact of light and alarm pheromone on immediate early gene expression in the European honeybee, *Apis mellifera. Entomological Science*					
Contribution	**Author initials;** decreasing order of responsibility				
Idea & study design	FMJS	JS			
Data collection	FMJS				
Data analysis & interpretation	FMJS				
Manuscript writing	FMJS	JS	WR		

Manuscript II (chapter V): Sommerlandt FMJ, Spaethe J. Rössler W, Dyer AG. (submitted). Does fine color discrimination learning in free-flying honeybees change mushroom body calyx neuroarchitecture?					
Contribution	**Author initials;** decreasing order of responsibility				
Idea & study design	FMJS	AGD	JS		
Data collection	FMJS	AGD			
Data analysis & interpretation	FMJS				
Manuscript writing	FMJS	JS	WR	AGD	

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Curriculum vitae

Place Date Signature

Publications in peer-reviewed journals

7. **Sommerlandt FMJ**, Spaethe J, Rössler W, Dyer AG [submitted]. Does fine color discrimination learning in free-flying honeybees change mushroom-body calyx neuroarchitecture? *PLoS ONE*

6. **Sommerlandt FMJ**, Rössler W, Spaethe J [in revision]. Impact of light and alarm pheromone on immediate early gene expression in the European honeybee, *Apis mellifera*. *Entomological Science*

5. Lichtenstein L, **Sommerlandt FMJ**, Spaethe J. (2015) Dumb and lazy? A comparison of color learning and memory retrieval in drones and workers of the buff-tailed bumblebee, *Bombus terrestris*, by means of PER conditioning. *PLoS ONE* **10**(7) DOI: 10.1371/journal.pone.0134248

4. Gutknecht L, Popp S, Waider J, **Sommerlandt FMJ**, Göppner C, Post A, Reif A, van den Hove D, Strekalova T, Schmitt AG, Colaco MB, Sommer C, Palme R, Lesch KP (2015). Interaction of brain 5-HT synthesis deficiency, chronic stress and sex differentially impact emotional behavior in Tph2 knockout mice. *Psychopharmacology* **232**(14) DOI: 10.1007/s00213-015-3879-0

3. **Sommerlandt FMJ**, Huber W, Spaethe J (2014). Social information in the stingless bee, *Trigona corvina* Cockerell (Hymenoptera: Apidae): The use of visual and olfactory cues at the food site. *Sociobiology* **61**: 401-406

2. **Sommerlandt FMJ**, Rössler W, Spaethe J (2014). Elemental and non-elemental olfactory learning using PER conditioning in the bumblebee, *Bombus terrestris*. *Apidologie* **45**(1) DOI: 10.1007/s13592-013-0227-4

1. Gutknecht L, Araragi N, Merker S, Waider J, **Sommerlandt FMJ**, Mlinar B, Baccini G, Mayer U, Proft F, Hamon M, Schmitt AG, Corradetti R, Lanfumey L, Lesch KP (2012). Impacts of Brain Serotonin Deficiency following Tph2 Inactivation on Development and Raphe Neuron Serotonergic Specification. *PLoS ONE* **7**(8): e43157.