Mechanisms and consequences of environmentally and behaviorally induced synaptic plasticity in the honey bee brain

Mechanismen und Konsequenzen umwelt- und verhaltensbedingter synaptischer Plastizität im Gehirn der Honigbiene

Doctoral thesis for a doctoral degree

at the Graduate School of Life Sciences,

Julius-Maximilians-Universität Würzburg,

Section Integrative Biology

submitted by

Nils Becker

from

Herford

Würzburg 2016

Submitted on: ……..……..

Members of the *Promotionskomitee*:

Chairperson:	**Prof. Dr. Thomas Dandekar**
Primary Supervisor:	**Prof. Dr. Wolfgang Rössler**
Supervisor (Second):	**Prof. Dr. Christian Stigloher**
Supervisor (Third):	**Prof. Dr. Ryszard Maleszka**

Date of Public Defence: ……..

Date of Receipt of Certificates: ……

Affidavit

I hereby confirm that my thesis entitled “Mechanisms and consequences of environmentally and behaviorally induced synaptic plasticity in the honey bee brain” 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.

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation “ Mechanismen und Konsequenzen umwelt- und verhaltensbedingter synaptischer Plastizität im Gehirn der Honigbiene“ 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.

Ort, Datum Unterschrift

Table of Contents

Summary

The brain is the central organ of an animal controlling its behavior. It integrates internal information from the body and external stimuli from the surrounding environment to mediate an appropriate behavioral response. Since the environment is constantly changing, a flexible adjustment of the brain to new conditions is crucial for the animals' fitness. The ability of the nervous system to adapt to new challenges is defined as plasticity. Over the last few decades great advances have been made in understanding the cellular and molecular mechanisms underlying neuronal plasticity. Plasticity may refer to structural changes physically remodeling the neuronal circuit, or to functional adaptations which are manifested in modified synaptic transmission, and in altered response and firing properties of single neurons. These structural and functional modifications are mediated by a complex interplay of environmental stimuli, intracellular signal transduction cascades, protein modifications, gene translation and transcription, and epigenetic gene regulatory mechanisms. However, especially the molecular mechanisms of environmentally-induced structural neuronal plasticity are still poorly understood.

In this thesis the honey bee was used as an innovative model organism to investigate this issue. The honey bee with its rich behavioral repertoire, highly sophisticated and plastic neuronal system, sequenced genome and full epigenetic machinery is well suited for studying the molecular underpinnings of environmentally-induced neuronal plasticity. Adult honey bees progress through a series of tasks within the dark hive until after about three weeks they start with foraging activities in the external world. The transition from in-hive to outside tasks is associated with remarkable structural neuronal plasticity. Subdivisions of the mushroom body, a brain region related to higher cognitive functions, are increased in volume. The volume expansion is mediated by a remarkable outgrowth of the dendritic network of mushroom body intrinsic neurons, so called Kenyon cells. In parallel, prominent synaptic structures, referred to as microglomeruli, are pruned. Most interestingly for this thesis, the pruning of microglomeruli and the dendritic expansion in Kenyon cells can be induced by a simple light exposure paradigm.

In the first chapter of the present thesis I used this paradigm to induce synaptic plasticity in the mushroom bodies under controlled lab conditions to search for correlating molecular changes which possibly mediate the observed plasticity. I compared the brain transcriptome of light-exposed and dark-kept control bees by whole transcriptome sequencing. This revealed a list of differentially expressed genes (DEGs). The list contains conserved genes which have reported functions in neuronal plasticity, thereby introducing them as candidate genes for plasticity in the honey bee brain. Furthermore, with this transcriptomic approach I discovered many candidate genes with unknown functions or functions so far unrelated to neuronal plasticity suggesting that these novel

genes may have yet unrecognized roles in neuronal plasticity. A number of DEGs are known to be methylated or to exert epigenetic modifications on themselves speaking for a strong impact of epigenetic mechanisms in light-induced structural plasticity in the honey bee brain. This notion is supported by a differential methylation pattern of one examined DEG between light-exposed and dark-kept bees as shown in this thesis. Also a plasticity-related microRNA, which is predicted to target genes associated with cytoskeleton formation, was found to be upregulated in light-exposed bees. This speaks for a translation regulatory mechanism in structural plasticity in the honey bee.

Another interesting outcome of this study is the age-dependent expression of DEGs. For some plasticity-related DEGs, the amplitude of light-induced expression differs between one- and seven-day-old bees, and also the basal expression level of many DEGs in naive dark-kept control bees significantly varies between the two age groups. This suggests that the responsiveness of plasticity-related genes to environmental stimuli is also under developmental (age-dependent) control, which may be important for normal maturation and for the regulation of age-related changes in behavior. Indeed, I was able to demonstrate in phototaxis experiments that one- and seven-day-old bees show different behaviors in response to light exposure and thus the correlating age-dependent transcriptional differences may serve as mechanisms promoting age-related changes in behavior.

Together the results of the transcriptomic study demonstrate the successfulness of my approach to identify candidate molecular mechanisms for environmentally-induced structural plasticity in the honey bee brain. Furthermore, the thesis provides seminal evidence for the implication of DNA methylation in this process.

To better understand the role of DNA methylation for neuronal and behavioral plasticity in the honey bee, the second chapter of the thesis aims at characterizing this molecular process under more natural conditions. Therefore, I examined the expression of the *DNA methyltransferase 3* (*DNMT3*) and of *Ten-eleven translocation methylcytosine dioxygenase* (*TET*) between in-hive bees and foragers. DNMT3 is responsible for DNA *de novo* methylation, whereas TET promotes DNA demethylation by converting methylcytosine (5mC) to hydroxymethylcytosine (5hmC). The data suggest that age and experience determine the expression of these two epigenetic key genes. Additionally, in this context, two examined DEGs are shown to be differentially methylated between nurses and foragers. One of these two DEGs, the plasticity related gene *bubblegum* (*bgm*), also exhibits an altered DNA methylation pattern in response to light exposure. Hence, these results of my thesis provide additional evidence for the importance of DNA methylation in behavioral and neuronal plasticity.

Results from the second chapter of this thesis also suggest additional functions of *DNMT3* and *TET* to their traditional roles in DNA methylation/demethylation. I show that *TET* is far more expressed in the honey bee brain than *DNMT3*. This stands in contrast to the relative scarcity of 5hmC compared to 5mC and points at extra functions of this gene like RNA modifications as reported for *Drosophila*. Antibody staining against the *DNMT3* gene product revealed an unexpected rare localization of the enzyme in the nucleus, but a surprisingly high abundance in the cytoplasm. The role of cytoplasmic DNMT3 is unknown. One possibility for the high abundance in the cytoplasm is a regulatory mechanism for DNA methylation by cytoplasmic-nuclear trafficking, or an additional function of DNMT3 in RNA modification, similar to TET.

Altogether, this thesis points at future research directions for neuronal plasticity by providing promising evidence for the involvement of epigenetic mechanisms and of a number of new candidate genes in environmentally induced structural plasticity in the honey bee brain. Furthermore, I present data suggesting so far unrecognized functions of DNMT3 which certainly need to be experimentally addressed in the future to fully understand the role of this enzyme.

Zusammenfassung

Das Gehirn ist das zentrale Organ zur Steuerung des Verhaltens von Tieren. Es integriert körperinterne Informationen mit Umweltreizen und sorgt somit für eine Anpassung des Verhaltens an die jeweilige Situation. Diese hohe Flexibilität des Gehirns ist ein entscheidender Überlebensfaktor in einer sich ständig wandelnden Umwelt. Die Fähigkeit des Nervensystems, sich an wechselnde Bedingungen anpassen zu können, wird als Plastizität bezeichnet. In den vergangen Jahrzehnten wurden große Fortschritte zur Identifikation von zellulären und molekularen Mechanismen neuronaler Plastizität erzielt. Neuronale Plastizität beinhaltet zum einen physische Veränderung der Struktur neuronaler Netzwerke, und zum anderen funktionale Modifikationen der internen Antworteigenschaften und synaptischen Übertragung eines Neurons. Diese Veränderungen werden durch ein komplexes Zusammenspiel zwischen Umweltreizen, intrazellulären Signalkaskaden, Proteinmodifikationen, Gentranslation und -Transkription, sowie epigenetischer Genregulation gesteuert. Die genauen Mechanismen umweltbedingter struktureller Plastizität sind jedoch immer noch nicht vollständig geklärt.

In der vorliegenden Doktorarbeit bediene ich mich der Honigbiene als innovativen Modellorganismus, um dieses Thema zu untersuchen. Die Honigbiene eignet sich durch ihr vielfältiges und komplexes Verhalten, ihr hochentwickeltes und plastisches Nervensystem, ihr sequenziertes Genom, sowie durch den Besitz einer vollständig epigenetischen Maschinerie besonders gut zur Untersuchung der molekularen Mechanismen umweltbedingter neuronaler Plastizität. Honigbienen führen die ersten drei Wochen ihres Lebens verschiedene Arbeiten innerhalb des dunklen Bienenstocks aus, ehe sie anschließend für den Rest ihres Lebens nach Nahrung außerhalb des Stocks suchen. Der Wechsel vom Innen- zum Außendienst korreliert mit plastischen Veränderungen des Gehirns. Subkompartimente des Pilzkörpers, einer Hirnstruktur, die mit höherer Kognition assoziiert wird, nehmen im Volumen zu. Diese Volumensexpansion lässt sich durch eine wachsende dendritische Verzweigung von Kenyonzellen, welche die intrinsischen Neuronen der Pilzkörper darstellen, erklären. Gleichzeitig findet eine Eliminierung von synaptischen Komplexen, sogenannten Mikroglomeruli, statt. Besonders interessant für diese Doktorarbeit ist die Tatsache, dass die strukturelle Plastizität der Mikroglomeruli und der dendritischen Verzweigungen bereits durch ein einfaches Lichtexponierungsprotokoll induziert werden kann.

In dieser Doktorarbeit löse ich durch das Lichtprotokoll synaptische Plastizität unter standardisierten Laborbedingungen aus, um nach zugrundeliegenden molekularen Veränderungen während dieses Vorgangs zu suchen. Ein Vergleich des Transkriptoms zwischen lichtbehandelten und dunkel gehaltenen Bienen mittels Transkriptom-Sequenzierung beider Gruppen erbrachte mehrere differentiell exprimierte Gene (DEGs). Die Liste der DEGs enthält einige konservierte Gene, denen

bereits eine Funktion in neuronaler Plastizität nachgewiesen wurde und daher als Kandidatengene für umweltbedingte synaptische Plastizität in der Honigbiene infrage kommen. Außerdem konnten mehrere DEGs ohne bekannten Bezug zu neuronaler Plastizität, oder mit bisher unbekannter Funktion, identifiziert werden, was die Möglichkeit einer bisher unentdeckten Rolle dieser Gene in neuronaler Plastizität impliziert. Des Weiteren sind einige der DEGs in epigenetische Prozesse involviert und aus anderen Studien ist bekannt, dass weitere DEGs Methylierungen aufweisen. Dies weist auf einen starken Einfluss epigenetischer Prozesse bei lichtinduzierter struktureller Plastizität in der Honigbiene hin. Diese Vermutung wird durch die differentielle Methylierung eines plastizitäts-assoziierten DEGs zwischen lichtexponierten und dunkelgehalten Bienen bekräftigt. Ferner ist eine microRNA in lichtbehandelten Bienen hochreguliert, welche als vorhergesagte Ziele Gene zur Zytoskelettformation hat. Dies spricht für einen translationsregulatorischen Mechanismus im Zusammenhang mit struktureller Plastizität in der Honigbiene.

Ein weiteres interessantes Ergebnis dieser Arbeit ist der starke Alterseinfluss auf die Expression der identifizierten DEGs. Bei einigen DEGs ist die Amplitude der lichtinduzierten Expression bei sieben Tage alten Bienen höher als bei einem Tag alten, und das Grundexpressionsniveau vieler DEGs in den naiven, dunkel gehaltenen Kontrollbienen unterscheidet sich zwischen den beiden Altersklassen. Dies lässt vermuten, dass die Expression von Plastizitätsgenen als Antwort auf Umweltreize ebenfalls durch ein internes Entwicklungsprogramm beeinflusst wird, was wichtig für eine normale Reifung, sowie zur Regulation altersabhängigen Verhaltens sein könnte. Tatsächlich weist diese Arbeit bei Phototaxis-Experimenten Verhaltensunterschiede zwischen einen- und sieben Tage alten Bienen auf, welche mit transkriptionellen Unterschieden zwischen den beiden Altersklassen korrelieren, die somit wiederum als molekularer Mechanismus zur Steuerung des altersabhängigen Verhaltens in Frage kommen.

Zusammengefasst bestätigen die Ergebnisse aus den Transkriptomstudien den Erfolg meines experimentellen Ansatzes zur Identifikation molekularer Kandidatenmechanismen für umweltbedingte strukturelle Plastizität im Honigbienengehirn. Zudem liefern die Ergebnisse der Transkriptions-Sequenzierung einen deutlichen Hinweis auf den Einfluss von DNA-Methylierung auf strukturelle neuronale Plastizität.

Um die Rolle von DNA-Methylierung im Zusammenhang mit neuronaler- und Verhaltensplastizität besser zu verstehen, zielt das zweite Kapitel dieser Arbeit auf eine genauere Charakterisierung dieses epigenetischen Vorgangs in einem natürlicheren Kontext ab. Dazu wurde die Expression der *DNA Methyltransferase 3* (*DNMT3*) und von *Ten-eleven translocation Methylcytosine Dioxygenase* (*TET*) zwischen Bienen im Innen- und Außendienst verglichen. DNMT3 ist zuständig für die *de novo* DNA-Methylierung, wohingegen TET DNA durch die Konvertierung von methylierten Cytosin (5mC) in

hydroxymethyliertes Cytosin (5hmC) demethyliert. Die Ergebnisse dieses Versuchs deuten auf eine altersabhängige, aber auch auf eine umweltbedingte Expression dieser beiden epigenetischen Schlüsselgene hin. Zusätzlich sind zwei untersuchte DEGs in diesem Versuchsaufbau differentiell methyliert, wobei eines dieser Gene mit Plastizitätsbezug, *bubblegum* (*bgm*), bereits in den Lichtexperimenten als differentiell methyliert charakterisiert wurde. Dadurch wird der Verdacht des Einflusses von DNA-Methylierung bei neuronaler- und Verhaltensplastizität weiter verstärkt.

Resultate der Versuche im zweiten Kapitel lassen weitere Funktionen von DNMT3 und TET über ihre traditionelle Rolle in DNA-Methylierung/Demethylierung hinaus vermuten. In dieser Arbeit ist *TET* im Honigbienengehirn weit mehr exprimiert als *DNMT3*. Das steht in starkem Kontrast zu dem relativ geringen Vorkommen von 5hmC im Vergleich zu 5mC und deutet auf zusätzliche Funktionen von TET in der Honigbiene hin, z.B. bei RNA-Modifikationen wie es bei *Drosophila* nachgewiesen wurde. Eine Antikörperfärbung gegen DNMT3 zeigt eine unerwartet geringe Konzentration dieses Enzyms im Zellkern, dafür aber ein relativ starkes Vorkommen im Zytoplasma. Die Funktion von zytoplasmatischem DNMT3 ist unbekannt, könnte aber einen regulatorischen Mechanismus der DNA-Methylierung durch zytoplasmatisch-nukleare Translokation des Enzyms darstellen, oder aber auf eine zusätzliche Rolle des Proteins bei RNA-Modifikationen, ähnlich wie für TET, hinweisen.

Abschließend lässt sich sagen, dass diese Doktorarbeit, durch die vielversprechende Identifizierung von epigenetischen Mechanismen und von aussichtsreichen Kandidatengenen für strukturelle Plastizität im Honigbienengehirn, auf neue Wege in der zukünftigen Erforschung neuronaler Plastizität weist. Des Weiteren präsentiere ich Daten, welche auf bisher unbekannte Funktionen von DNMT3 hinweisen und eine weitere Erforschung dieses Enzyms nötig machen um seine Rolle vollständig zu verstehen.

General Introduction

More than 100 years ago, the famous neuroscientist Ramón y Cajal recognized that the brain is built of a gigantic number of distinct nerve cells. Most nerve cells have a characteristic polarized shape formed by separate neuronal processes termed axons and dendrites. Axons connect to the dendrites of other cells by synapses. Y Cajal expressed the idea that the wiring of the brain is not fixed, but rather plastic and that the synaptic connections could be modified by experience (y Cajal, 1928). The last few decades of modern research proved y Cajal right by revealing many examples for the astonishing high flexibility of the brain in animal species ranging, for example, from mammals to honey bees. But what exactly causes neuronal plasticity? Which forms of plasticity do exist? How are they mediated? And what are their functional and behavioral consequences? The search for answers to these fundamental questions aiming at the underlying mechanisms of neuronal plasticity is still at the beginning. In the first part of the introduction of this thesis I will give a brief overview about advances made in unraveling these questions, and in the second part I present the honey bee as a well suited model organism to address this topic.

Neuronal plasticity

The brain is the central organ controlling an animal's interaction with the environment. It integrates internal states with external visual, auditory, tactile, olfactory and gustatory stimuli to determine an appropriate behavioral response. The environment is constantly changing and, therefore, it is not surprising that the nervous system is subject to remarkable adjustments. The ability of the nervous system to adapt to various functional challenges throughout lifetime is defined as plasticity (Zilles, 1992).

Forms of neuronal plasticity

Plasticity can be generally categorized in three, partly overlapping, types: experience-independent plasticity, experience-expectant plasticity, and experience-dependent plasticity (Greenough et al. 1987; Kolb & Gibb 2014). Experience-independent and -expectant plasticity mainly occur during development and are characterized by a refinement and pruning of the innate overproduction of synaptic connections (Changeux and Danchin, 1976; Cowan et al., 1984). These two forms of plasticity differ from experience-dependent plasticity in the way that experience-dependent plasticity incorporates events that are unique to each individual, such as learning a specific language. In the other two forms events are processed which are stereotyped or basic and common to each individual like the basic elements of pattern perception (Greenough et al., 1987; Kolb and Gibb, 2014).

In experience-independent plasticity, a selection of connections takes place driven by stereotyped, internal events. For example, in the visual system of the cat the lateral geniculate nucleus (LGN) exhibits layers which are exclusively innervated by axons of only one eye. However, during prenatal development layers of the LGN also receive inappropriate, minor connections from the other eye. Through spontaneous internal and synchronized firing of axons from the appropriate eye the corresponding innervations in the LGN are strengthened, whereas connections from out of synchronization firing axons from the other eye are weakened and finally pruned (Campbell and Shatz, 1992; Kolb and Gibb, 2014).

An example for experience-expectant plasticity also comes from the visual system of the cat. The visual cortex comprises alternating ocular dominance columns which have a preferred responsiveness to input of either one eye or the other. If a kitten is monocular deprived after birth for several weeks, the open eye will expand its representation in the visual cortex while columns of the closed eye diminish. Is the deprived eye re-opened, vision will be comprised, and that is why the period in which experience is expected and required for normal development is often referred to as critical or sensitive period (Wiesel and Hubel, 1963; Campbell and Shatz, 1992; Hensch, 2005; Kolb and Gibb, 2014).

While experience-independent and-expectant plasticity are important for the normal development of brain circuits, experience-dependent plasticity shapes existing circuits in response to the specific environmental information the individual is exposed to. Experience-dependent plasticity can be caused by various different factors. In the 1960's Rosenzweig and colleagues were the first to report plasticity in the rat brain following an enriched environment (Rosenzweig et al., 1962). Rats or mice from cages with frequently changed toys have increased brain weight and a thicker cortex compared to conspecifics in standard cages (Bennett et al., 1964; Anderson, 2011). These modifications are correlated with dendritic branching and variation of synapse number (Kolb, 1998). Similar effects on the brain can be observed after more specific tasks like motor- and spatial learning (Kleim et al., 1998; Kolb et al., 2008). Although most research has been conducted in mammals, invertebrates have also been shown to be subject to astonishing experience-dependent plasticity. In the snail *Aplysia* stimulation of the tail causes the retraction of the gill and siphon. Repeated electric shocks to the tail lead to an increased response of the gill- and siphon withdrawal reflex, which persists even for harmless, moderate stimuli. The sensitization of this reflex is accompanied by the enhanced excitability of existing synapses, as well as with the formation of new synapses of the involved sensory neurons (Antonov et al., 2003; Bailey and Kandel, 2008). The number of neuronal fibers and volumes of brain compartments in *Drosophila* depends on the rearing conditions with conspecifics. Female flies grown in a dense population have increased fiber numbers compared to flies grown in

low density cultures. When kept pairwise, flies have larger brain sub-volumes with a female partner than with a male partner (Heisenberg et al., 1995). In the carpenter ant *Camponotus floridanus*, brain sub-volumes are influenced by task. Ants performing foraging duties outside the nest have enlarged sub-divisions of the brain compared to age-matched individuals committed to inside-tasks (Gronenberg et al., 1996).

Together these examples demonstrate that various forms of neuronal plasticity occur in diverse species. The underlying mechanism of neuronal plasticity can be divided in a few categories either related to structural or non-structural changes (Fig. 1).

Non-structural changes, often termed functional changes, include Hebbian and intrinsic plasticity, which influence the properties of existing structures. Hebbian plasticity describes long-term potentiation (LTP) or a long-term depression (LTD) of the postsynaptic response after repeated stimulation by the presynapse. The increased or decreased synaptic strength is mediated by neurotransmitter receptors. Calcium influx in the postsynapses induces an intracellular signal cascade ultimately leading to the incorporation of more neurotransmitter receptors in the postsynaptic membrane for LTP, or to a reduction of receptors for LTD (Malenka and Bear, 2004).

Intrinsic plasticity refers to non-synaptic adaptations of the excitability of a neuron which involves the modification of passive and active membrane properties determining the action potential. The intrinsic properties of the neuron are tuned by regulating the expression or biophysical features of ion channels in dendrites and axons (Mozzachiodi and Byrne, 2010; Guzman-Karlsson et al., 2014). Pairing a light stimulus with physical rotation led to increased response latencies of the mollusk *Hermissenda* to enter an illuminated area. The changes in phototactic behavior are correlated with increased spontaneous firing and input resistance, due to changed potassium currents, in specific photoreceptors (Crow and Alkon, 1980; Farley, 1988).

Structural neuronal plasticity upon experience is expressed in the dynamics of axonal and dendritical arbors, bouton and spine growth and elimination, and finally synapse growth and elimination. In the mouse and rat hippocampus it could be shown that environmental enrichment and spatial learning lead to an expansion of axonal terminal arborizations (Galimberti et al., 2006; Holahan et al., 2006). Dendritic arborization and length also increased in learning tasks in rats (Kolb et al., 2008).

Remodeling of axonal and dendritic branches is often associated with a turnover of boutons and spines, and therefore likely to influence synapse growth or elimination (De Paola et al., 2006). Boutons are small excesses found at the length of the axon, or at its terminal end. Synapses are formed by contacts of these axonal boutons with small dendritic protrusion, so-called dendritic spines. However, a small number of boutons and spines do not localize to synapses, but may exist

independently. The appearing and disappearing of boutons and spines occurs at higher rates in young mice and decreases in mature adults (Holtmaat and Svoboda, 2009). The turnover may be influenced by experience (Holtmaat et al., 2006; Holtmaat and Svoboda, 2009; Xu et al., 2009).

The growth of new spines often leads to the formation of new synapses. In the somatosensory cortex of mice it has been demonstrated that spines form new synaptic connections preferably at boutons which already have multiple synapses, rather than at *de novo* boutons (Knott et al., 2006). Associative learning was shown to lead to an increase of multi-synapse boutons in rabbits (Geinisman et al., 2001). In contrast, the induction of LTD stimulates the elimination of synapses in rat hippocampus slices by spatially disaggregating spines and boutons without eliminating these structures themselves (Bastrikova et al., 2008). The transition from in-nest to foraging activities of the desert ant *Cataglyphis fortis* is accompanied by a loss of synapses due to presynaptic pruning of projection neuron boutons, and, at the same time, growth of dendrites (Stieb et al., 2010).

There is evidence that the different forms of neuronal plasticity promote each other and work in parallel and coherently. For example, changes in synaptic efficacy in terms of LTP and LTD are often accompanied by plasticity of intrinsic neuronal excitability. This synaptic and non-synaptic plasticity is thought to work in synergy to modulate neuronal output (Debanne, 2010). Furthermore, LTP induction was shown to change spine morphology and thereby strengthening synapses or even facilitating the growth of new spines (Matsuzaki et al., 2004; Nägerl et al., 2004; Meyer et al., 2014). LTD causes weakening of synapses which may fall apart again by elimination of boutons (Nägerl et al., 2004; Becker et al., 2008). Another example comes from the arborization of axons which often leads to the development of new boutons promoting the formation of new synapses (De Paola et al., 2006).

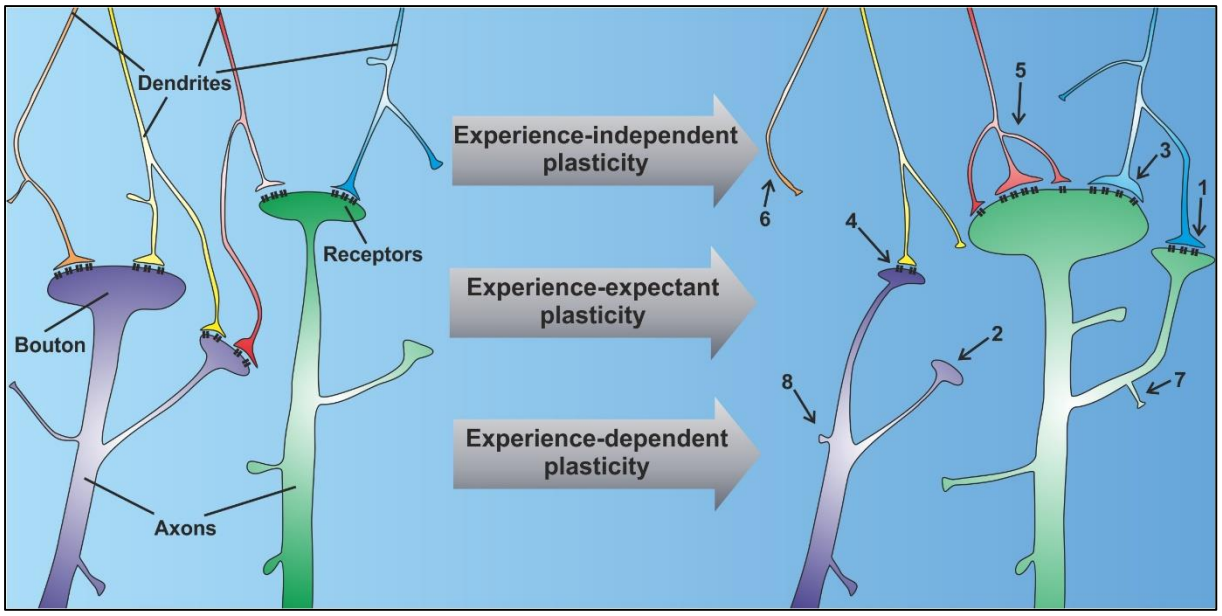


Figure 1: Forms of neuronal plasticity. 1, Synapse formation; 2, Synapse elimination; 3, Synapse strengthening (synapse enlargement, LTP); 4, Synapse weakening (synapse shrinkage, LTD); 5, Dendritic outgrowth/branching; 6, Dendritic retraction; 7, Axonal outgrowth/branching; 8, Axonal retraction.

Molecular mechanism underlying neuronal plasticity

The vast majority of research on the molecular mechanism of neuronal plasticity has been performed in the context of functional changes (LTP/LTD) as well as in a development context. Although there are likely to be mechanistic differences between plasticity in developing and mature organisms, recent work investigating experience-dependent plasticity in mature animals provides evidence for a great overlap of the molecular underpinning between these various forms of plasticity (Hu et al., 2013). A great number of genes and proteins is involved in shaping the neuronal circuit and only a few prominent key molecules illustrating fundamental principles shall be mentioned here.

The molecular mechanisms involved in neuronal plasticity can be roughly divided in three categories according to their temporal occurrence. First, rapid and short-term changes upon neuronal activity lasting for a couple of hours involve enhanced neurotransmitter release (Bayazitov et al., 2007), activation of kinases (Lisman et al., 2012), and trafficking of the glutamatergic receptors AMPA and NMDAR to or from synaptic membranes (Malenka and Bear, 2004). Second, intermediate long changes in synaptic strength and structure lasting for hours to days additionally relay on protein translation from a pool of local synaptic mRNAs (Sutton and Schuman, 2006; Briz et al., 2015). Finally, long-term functional and structural changes are mediated by new gene transcription (Flavell and Greenberg, 2008; West and Greenberg, 2011; Ryan et al., 2015).

The cytoskeleton plays a central role in these three phases of plasticity (Spence and Soderling, 2015). Dendritic- , and to some extent, axonal protrusions with the potential to form synapses consist of a dense, filamentous actin (f-actin) network assembled from a pool of monomeric actin molecules (G-actin). F-actin controls spine formation and maintenance, synaptic plasticity, spine turnover, and

morphological reshaping in response to environmental factors (Holtmaat and Svoboda, 2009; Spence and Soderling, 2015). The assembly of actins is regulated by three types of proteins: Arp 2/3, formins, and profilin. For the formation of dense, branched networks, the activation of Arp 2/3 is required by nucleation promoting factors such as N-WASP, WAVE1, or WASH. Conditional Arp 2/3 knockout mice exhibit abnormal spine morphologies which correlates with disturbed cognition (Kim et al., 2013). Arp 2/3 also is involved in axonal branching of sensory neurons and growth of filopodia (Kalil and Dent, 2013). Filopodia are tiny precursors of axonal branches and dendritic spines. Formins like FMN2 promote the growth of filopodia by facilitating linear actin polymerization. Loss of FMN2 in mice results in a reduced number of spines and deficits in fear-learning (Law et al., 2014). Profilin is important for recruiting G-actin to allow actin polymerization and spine reshaping upon neuronal activity (Ackermann and Matus, 2003). Furthermore, the recruitment of profilin to dendrites could be demonstrated upon fear-learning (Lamprecht et al., 2006). The disassembly of f-actin is mediated by actin-depolimerizing factors (ADF) and cofilins (Bernstein and Bamburg, 2010). The pushing force of f-actin is necessary for initial membrane protrusions, but also a second cytoskeleton structure, microtubules, play an important role in axonal branching, as only filopodia containing microtubules develop into branches (Dent et al., 1999). The coordination of actin and microtubule dynamics is thought to be essential in this process and is mediated by septin proteins that interact with both cytoskeleton components. SEPT7 promotes the entry of microtubules into filopodia (Hu et al., 2012).

Phosphorylation events resemble an important factor in neuronal plasticity. For example, cofilin can be inactivated by phosphorylation by the Lim kinase 1 (LIMK-1) (Yang et al., 1998). Knock-out mice for LIMK-1 have abnormalities in spine shape, exhibit enhanced LTP, and cognitive deficits (Meng et al., 2002). The action of the above introduced cytoskeleton regulatory proteins is modulated by Rho family GTPases such as Rho, Rac, and Cdc24 (Spence and Soderling, 2015). RhoA has also been associated with axonal branching (Ohnami et al., 2008). GTPases in turn are recruited by phosphorylation through the calcium sensitive kinase CamKII. CamKII serves as an calcium sensor which is activated upon activity-dependent local calcium influx into synaptic structures (Murakoshi et al., 2011). Activated CamKII results in synapse strengthening and enlargement (Lisman et al., 2012). Another important kinase in synaptic structures is PKC. The cytoskeleton stabilizing protein β-adducin is necessary for the maintenance of newly formed synapses and its phosphorylation through PKC mediates synapse disassembly following environmental enrichment (Bednarek and Caroni, 2011).

Contacts between filopodia and axons that eventually result in spine- and subsequently in synapse formation are established by trans-synaptic adhesion proteins. An example for such a protein is the transmembrane protein cadherin which couples with its intracellular tail to f-actin via catenins. Catenins are thought to mediate activity-dependent changes of the cytoskeleton during synaptic

plasticity (Abe et al., 2004). Another synaptic cell adhesion molecule, SYG-1, is required for synapse formation and axonal branching by mediating the assembly of an f-actin network at presynaptic sites through interaction with the WVE-1/WAVE regulatory complex (Chia et al., 2014).

Once synapses have been formed, the synapse is subject to remodeling in response to presynaptic input which may result in LTP or LTD. Functional changes relay on glutamate receptors such as AMPAR and NMDAR. Trafficking of these receptors is heavily influenced by the actin cytoskeleton machinery which recruits the receptors to the membrane or mediates their endocytosis from there (Spence and Soderling, 2015).

The consolidation of LTP/LTD and also some forms of structural changes are dependent on local protein translation in synaptic structures. In mammals, signaling from metabotropic glutamate receptors lead to dendritic protein translation, formation of LTD and elongation of dendritic spines which depend on local protein synthesis (Sutton and Schuman, 2006). In *Aplysia*, long-term facilitation of synapses between sensory and motor neurons leads to growth of new sensory neuron varicosities, which relies on local protein synthesis and strengthens the synapse. Blocking of local protein translation interrupts these changes (Sutton and Schuman, 2006). The mechanisms of local protein synthesis dependent plasticity are yet not fully understood and only a handful of locally translated proteins upon neuronal activity could be identified so far. These include CaMKII, a isoform of PKC (PKMζ), activity-regulated cytoskeleton-associated protein (Arc), and RhoA (Sutton and Schuman, 2006; Messaoudi et al., 2007; Briz et al., 2015).

In addition to local protein synthesis, neuronal-activity can evoke new gene transcription underlying neuronal plasticity. Neurotransmitters released by the presynapse excite postsynaptic receptors which mediate calcium influx across the membrane of the neuron leading to an elevation of cytoplasmic but also nuclear calcium levels. This influx then activates calcium sensitive enzymes such as CamKI/II, MAPK or the calcium-regulated phosphatase calcineurin (West and Greenberg, 2011). For example, CamkI phosphorylates the transcription factor CREB which leads to dendrite arborization. The dendritic outgrowth is mediated by CREB-dependent transcription of Wnt-2, a protein implicated in dendrite development (Wayman et al., 2006).

Next to transcription factors, epigenetic mechanisms such as microRNA (miRNA) modulations, histone- and DNA modifications serve to regulate transcription and translation of neuronal plasticity genes (West and Greenberg, 2011). miRNAs are small approx. 20 nt long RNAs which bind to mRNAs thereby blocking their translation. An example for this is the CREB-regulated miRNA miR132. miR132 hampers translation of *p250GAP* thereby modulating the p250GAP-Rac-Pak signaling cascade. Inhibition of *p250GAP* leads to increased spine formation (Impey et al., 2010).

Histones are built of eight subunits (two copies of each H2a, H2b, H3, H4) around which a 147 bp long stretch of DNA wraps, together forming the nucleosomes that make up the chromatin. Posttranslational histone modifications at the amino-terminal tail domains of histones such as methylation, acetylation, and phosphorylation modulate the strength of the DNA - histone interaction and thereby the accessibility of the DNA for gene transcription (Strahl and Allis, 2000). The enzymes responsible for the modifications respond to neuronal activity and especially histone acetylation is linked to transcriptional activity which determines synaptic plasticity (West and Greenberg, 2011). *C-Fos* gives an example for transcription regulated by histone modifications. *C-Fos* belongs to the immediate early genes which are transcribed quickly after neuronal activity and serve as transcription factors. *C-Fos* positively regulates synaptic strength and synapse numbers (Sanyal et al., 2002; Fleischmann et al., 2003). In resting neurons, the transcription of *c-fos* is blocked by histone deacetylation mediated by histone deacytylases (HDACs) (Qiu and Ghosh, 2008). Upon activity, the histone acetyltransferase CBP is recruited to the promoter of *c-fos* and HDACs are phosphorylated which promotes their export out of the nucleus. These events lead to the acetylation at the *c-fos* promotor and subsequently to its transcription (West and Greenberg, 2011).

Recent studies give evidence for the role of DNA methylation as another form of epigenetic chromatin remodeling in neuronal plasticity. DNA methyl transferases (DNMTs) thereby methylate single DNA cytosines in a CpG context to form 5-methylcytosines (5mC) (Jurkowska et al., 2011a). The erasure of the methyl marks is mediated by TET enzymes which convert 5mC into 5-hydroxymethylcytosine (5hmC) (Bhutani et al., 2011). The involvement of DNA methylation in neuronal changes is supported by the notion that neuronal activity changes DNA methylation of genes related to neuronal plasticity, which partly negatively correlates with their expression (Guo et al., 2011). In rats, induction of LTP and fear conditioning leads to an increase of DNMT levels, whereas *reelin*, a gene encoding an enzyme involved in spine formation, is demethylated. This indicates that DNA methylation as well as demethylation are important for neuronal plasticity (Miller and Sweatt, 2007; Sui et al., 2012; Kim et al., 2015). Knockout of *DNMT1* and *DNMT3* in mice induced the upregulation of the plasticity-related genes and led to deficits in learning and memory, accompanied by decreased volumes of the hippocampus due to smaller neurons (Feng et al., 2010). Furthermore, changes in synaptic strength depend on demethylation events by TET1 by affecting glutamatergic responsiveness (Meadows et al., 2015).

The honey bee as a model organism for neuronal plasticity

Many aspects of the molecular mechanisms of neuronal plasticity and their consequences on the neuronal network and subsequently on behavior are still not very well understood. The honey bee with its rich behavioral repertoire, its sophisticated and highly plastic neuronal system, and,

moreover, its sequenced genome and full representation of the epigenetic machinery is an valuable model organism for the unanswered questions of neuronal plasticity (Wang et al., 2006; Weinstock et al., 2006).

The life of the honey bee

Highly social insects like ants, wasps, and bees are found in nearly all habitats around the world. They make up an enormous amount of the total animal biomass that can be as high as 80% in tropical regions (Hölldobler and Wilson, 2009). The extreme ecological success can be attributed to the insects' eusocial lifestyle which helps them to cooperatively sustain the colony. Eusociality is defined by division of labor into reproductive and non-reproductive groups, overlapping adult generations, and cooperative brood care (Wilson, 1971). In a typical honey bee colony, eusociality is manifested by one reproductive queen, several hundred male drones, as well as by 30,000 – 50,000 sterile female workers (Winston, 1991). Drones are only produced by the colony during the mating season and serve the sole function to reproduce with queens produced by other colonies. After this defined time, they become useless for the colony and are abandoned from the hive. Queens commit to only one task: laying eggs and fertilizing them with sperm received from drones during the maiden flight and stored in spermatheca. But not all eggs are fertilized. Males derive from unfertilized eggs, which makes them haploid, whereas females develop from fertilized eggs resulting either in diploid workers or queens. The outcome into polymorphic different workers or queens depends on differential feeding. Workers develop from larvae fed with pollen and honey while the production of queens is controlled by epigenetic mechanisms induced by prolonged feeding with royal jelly (Winston, 1991; Kucharski et al., 2008). As drones and queens are only involved in reproduction, the workers of the colony take over the vast majority of duties for the hive via an age-related division of labor (Robinson 1992). Young bees progress through a series of tasks inside the hive like cell cleaning, nursing the brood, receiving nectar, or building the comb (Fig. 2). As young bees predominantly commit to nursing the brood, they are commonly referred to as nurses. After about two to three weeks they engage in work with closer proximity to the hive entrance, which includes ventilating air into the hive or guarding the hive entrance. After three weeks, bees begin with first orientation flights to prepare themselves for foraging activities which they perform for their remaining life the next two to four weeks (Lindauer, 1952; Capaldi et al., 2000; Degen et al., 2015). This temporal polytheism is not strictly age-regulated but may be influenced by the needs of the colony. In case of a shortage of nurses, foragers may convert back to nursing tasks. Furthermore, experiments removing all foragers promote the premature transition from young bees into foragers (Robinson, 1992). Together these findings demonstrate the highly plastic behavior of the honey bee, whereby especially the drastic switch from inside to outside duties represents a remarkable behavioral challenge. While young bees act in the dark hive where they rely on mainly olfactory cues, foragers experience a completely

different environment in which visual stimuli become of great importance. Foragers need to navigate through a three dimensional landscape to food sources, sometimes several kilometers, and find their way back to the hive. This is accomplished by orientation based on the position of the sun, polarized sky light pattern, and landmarks, and requires the ability of learning and memory to be able to perform these complex navigational tasks (Dyer, 1987; Menzel et al., 2010). In addition to that, bees need to identify and discriminate potential food sources such as colored flowers and remember their location if they were profitable (Collett and Collett, 2002). Foragers are even able to communicate the location of attractive food sources to their sisters in the hive by a sophisticated dance language (Von Frisch, 1967). Thus, the transition from nursing to foraging is a highly demanding cognitive challenge, which is likely to be represented by adaptive changes of the brain.

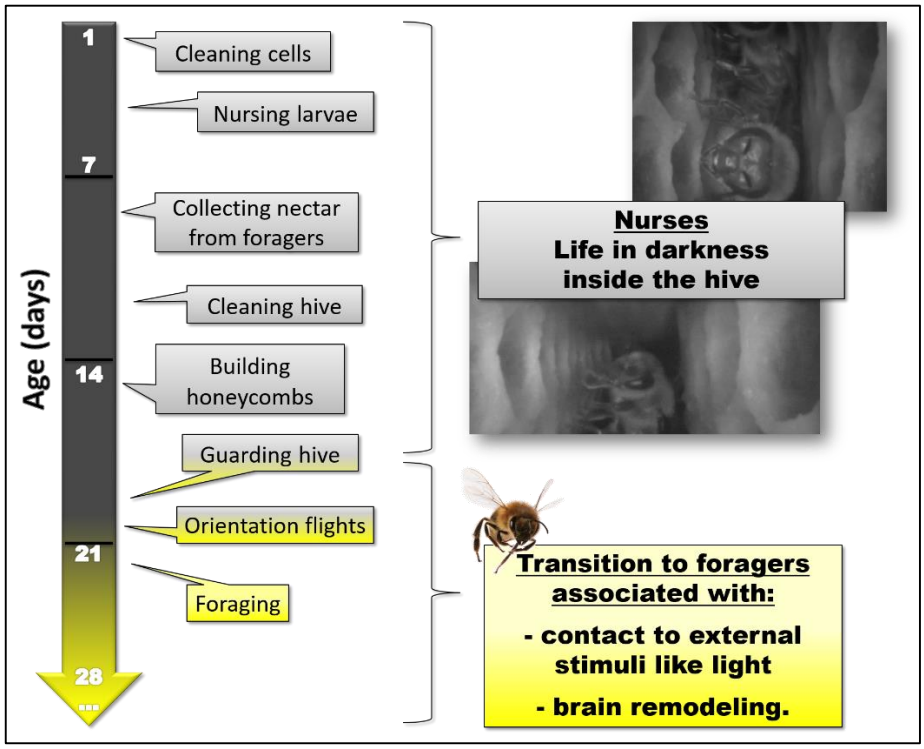


Figure 2: The life of the honey bee. Age-related polytheism is associated with neuronal plasticity.

Anatomy of prominent brain regions of the honey bee

The honey bee brain is subject to remarkable neuronal plasticity described especially for the antennal lobes (ALs), optic lobes (OLs), and mushroom bodies (MBs). First, the neuroanatomy of these three regions shall be roughly described before giving examples of their plasticity.

The symmetrical bilateral brain of the honey bee consists of approximately 960,000 neurons which are organized in distinct cell clusters and neuropils (Menzel & Giurfa 2001; Rössler & Groh 2012). The ventrally located deutocerebrum contains the ALs (Fig. 3). The ALs represent the primary antennal sensory centers which receive and process olfactory information. The stimuli are transferred by olfactory receptor neurons from the antenna to spherical subunits within the ALs, termed glomeruli,

where they connect with projection neurons which further convey the information via a dual pathway to higher order brain centers (Kirschner et al., 2006; Brill et al., 2013).

Dorsally of the deutocerebrum the protecerebrum is found including the central complex, lateral horn, protocerebral lobes, and the prominent OLs and MBs (Ito et al., 2014). The comparably large OLs receive and process visual information. The first neuropil of the OLs to which axons of photoreceptors connect is the lamina. Lamina axons project further to the medulla into distinct columns, which are interconnected by horizontal projections. The third and last neuropil of the OLs is the lobula receiving contacts from the medulla. Commissures connect both optic lobes from each hemisphere. Furthermore, the medulla and lobula form projections to the lateral protecerebrum and to the MBs (Avarguès-Weber et al., 2012).

The MBs of insects are considered as higher-order multi modal integration centers important for cognitive functions like learning and memory (Menzel and Giurfa, 2001; Fahrbach, 2006). They are located in the center of the brain, dorsally to the ALs and flanked by the OLs, and form a paired structure in each hemisphere. The structure is composed of two cup shaped calyces, one pedunculus, and the vertical and medial output lobes. This form, reminding of a mushroom, originates from about 368,000 MB intrinsic neurons, the Kenyon cells (Menzel and Giurfa, 2001; Rössler and Groh, 2012). The cell bodies of the Kenyon cells are located in the center and around the calyx whereas their dendritic arborizations form the typical cup-shaped neuropil in-between. The Kenyon cell axons finally build the pedunculus at the base of the calyx, where they bifurcate into the two main output regions, the vertical and medial lobe. The calyx with its intense dendritic arborizations represents the input region of the MBs and can be further subdivided into three regions according to their main input modality. The lip at the top of the calyx receives predominately olfactory projections, the collar bellow obtains visual input, and the basal ring contains connections from both modalities (Fahrbach, 2006). Within the calyx projections from sensory neurons form glomerular shaped synaptic connections, termed microglomeruli (MG), with Kenyon cell dendrites. A MG is composed by a presynaptic bouton in the center and surrounding dendritic profiles (Rössler and Groh, 2012). Depending on age, a bouton receives about 95 -140 dendritic contacts, with two to three contacts per active zone (Groh et al., 2012).

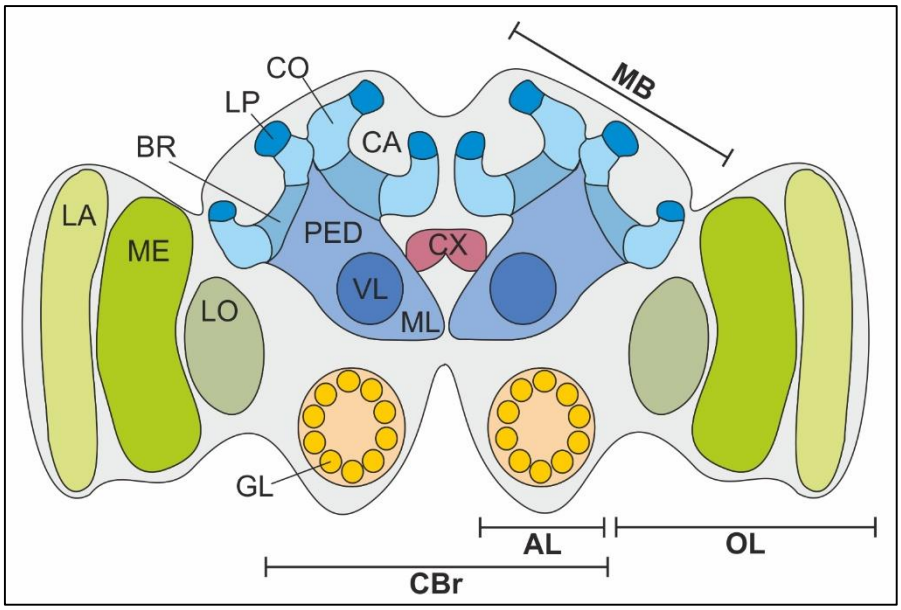


Figure 3: Schematic frontal view of a honey bee brain section. LA, Lamina; ME, Medulla; LO, Lobula; BR, Basal ring; LP, Lip; CO, Collar; CA, Calyx; PED, Pedunculus; VL, Ventral lobe; ML, Medial lobe; GL, Glomerulus; CX, Central complex; MB, Mushroom body; AL, Antennal lobe; OL, Optic lobe; CBr, Central brain.

Examples of neuronal plasticity of the honey bee brain

After eclosion, the MB calyces undergo a rapid volume increase until a plateau is reached at around day seven (Muenz et al., 2015). This volume increase even takes place in light-deprived and socially isolated bees and may thus be mediated by an internal program (Fahrbach et al., 1998). The volume increase is due to an extensive Kenyon-cell dendritic outgrowth (Farris et al., 2001; Muenz et al., 2015). From day seven on, the number of projection neuron boutons in the calyx declines, whereas the number of dendritic contacts per bouton, as well as the volume of the boutons, increases when comparing old and newly emerged bees (Groh et al., 2012; Muenz et al., 2015).

However, structural plasticity in the honey bee brain is not only age-related but may also be induced and influenced by behavioral and environmental factors. For example, the transition from nursing to foraging correlates with profound structural changes of the MBs which are likely to reflect adaptations to a new environment and behavior. Foragers have larger calyx volumes, and longer, more branched Kenyon-cell dendrites than age-matched nurses (Withers et al., 1993; Farris et al., 2001; Maleszka et al., 2009). Especially the collar volume is heavily affected by experience, which points out the new gain of importance of visual stimuli for foragers (Durst et al., 1994). The reduction of MG density observed in foragers compared to one-day-old bees (Groh et al., 2012) can also be evoked by artificial light exposure (Scholl et al., 2014). Three- and ten day-old bees, as well as winterbees, have reduced MG densities in the collar when exposed for three days to light pulses compared to a dark-kept control group. The reduction even seems to be reversible, as foragers kept in dark cages for three days had higher MG densities than their freely foraging age-matched conspecifics from the hive (Scholl et al., 2014). The social environment also influences the age-

related development of the MBs of adults in the first week of their life. Eight day-old bees isolated in cages have smaller MB volumes than age-matched bees from the hive, but when a dead bee is added to the cage the MB volume increase is even higher compared to the hive bees (Maleszka et al., 2009).

Neuro-structural plasticity has also been described in other brain areas than the MBs. Classical conditioning, where bees learn to associate an odor with a sugar reward, not only leads to increased MG densities in the lip of the MB, but also to a volume increase of some glomeruli of the ALs (Hourcade et al., 2009, 2010). The formation of long-term memory by this protocol is further reflected by an increased activity of MB output neurons to the rewarded stimulus (Strube-Bloss et al., 2011). Moreover, the glomerulus T1-44 was shown to be larger and to contain more synapses in foragers compared to younger nurses (Brown et al., 2002). In young bees, a premature increase of T1-44 volume can be induced by precocious foraging (Brown et al., 2004).

Finally, an elegant study could link manipulations of the visual environment to changes in phototactic behavior, which correlated with functional and structural plasticity of photoreceptor neurons in the lamina (Hertel, 1983). Bees reared under UV light, and therefore under long wavelength deprivation, are less sensitive to blue and green light in a phototactic experiment. Accordingly, electroretinogram responses to green light are altered. This correlates with a decreased number of synapses formed by green sensitive photoreceptors in the lamina.

Potential molecular mechanisms of neuronal plasticity in the honey bee

Despite the extensive structural plasticity shown in the honey bee brain upon natural behavioral development, learning and memory, and artificial stimulation, nearly nothing is known about the underlying molecular mechanisms. However, a variety of studies have investigated the molecular changes during the transition from nursing to foraging and identified a few molecules and processes related to neuronal plasticity which may therefore contribute to the architectural remodeling of the brain during this transition (Kucharski and Maleszka, 2002a; Lutz et al., 2012; Zayed and Robinson, 2012). Genes found to be upregulated in foragers compared to nurses include the genes *jib,* encoding an enzyme known to modulate MAPK signaling pathway, and *ino*s, encoding for an inositol-3-phosphatase associated with neuronal plasticity (Lutz et al., 2012). The transcription of inositol-triphosphat 3-kinases is also affected by the nurse-forager transition (Kucharski and Maleszka, 2002b). Furthermore, gene ontology enrichment analysis of genes induced by the transition revealed a clustering into the categories small GTPase mediated signal transduction, nuclear export, protein kinase activity, regulation of RNA splicing, plasma membrane part, actin cytoskeleton organization, and cell morphogenesis (Lutz et al., 2012). Accordingly, an activity increase of the small GTPase Rac, and a decrease of GTPase RhoA activity, was reported for experienced foragers versus new foragers (Dobrin and Fahrbach, 2012). Additionally, transcription factors like Creb are differentially expressed

in foragers compared to nurses (Chandrasekaran et al., 2011; Zayed and Robinson, 2012). Epigenetic mechanisms may also contribute to neuronal plasticity during the behavioral transition. A search for differentially methylated genes between nurses and foragers revealed many genes involved in helicase activity, chromatin remodeling, and neuronal development (Herb et al., 2012). The affected genes include *iswi*, which functions in dendritic outgrowth, and *Nadrin*, a Rho GTPase activating protein (Herb et al., 2012; Lockett et al., 2012). The upregulation of several miRNAs correlates with the behavioral change, including miR let-7, a miRNA involved in axonal plasticity (Behura and Whitfield, 2010; Zou et al., 2013).

Furthermore, many studies identified genes and proteins playing a role in learning and memory in the honey bee brain and, as it is widely accepted that learning leads to physical traces, these molecules might participate in structural changes of the brain. A remarkable overlap of the molecular mechanisms leading to memory formation exists between the honey bee and other vertebrate and non-vertebrate model organisms (Menzel, 2012). Four forms of memory which are mediated by distinct molecular pathways have been described in olfactory conditioning in the honey bee: short-term memory, middle-term memory, translational-dependent long-term memory, and finally translational- and transcriptional-dependent long-term memory (Menzel, 2012). Memory consolidation requires the action of the protein kinases PKM, PKC and PKA (Grünbaum and Müller, 1998; Friedrich, 2004). Creb and CamKII, known mediators of learning and memory in other model organisms, are also involved in learning and memory in the honey bee (Biergans et al., 2015; Scholl et al., 2015; Gehring et al., 2016). The transcription-dependent formation of olfactory long-term memory correlates with an increase in MG-density in the lip of the MBs (Hourcade et al., 2010). Chromatin remodeling also plays a central role in memory formation in the honey bee. Olfactory learning protocols induce changes in histone acetylation and inhibition of histone acetyl transferases or histone deacetylases lead to impairments in learning and memory (Merschbaecher et al., 2012, 2016; Lockett et al., 2014). DNA methylation and -demethylation is also involved. Olfactory learning promotes changes in the DNA-methylation pattern of memory-related genes and inhibition of DNMTs leads to reduced learning and memory ability (Lockett et al., 2010; Biergans et al., 2015). Finally, miRNAs were shown to contribute in visual and olfactory learning tasks (Cristino et al., 2014; Qin et al., 2014). The knockdown of miR-932, which targets actin, correlates with an impairment of long-term memory (Cristino et al., 2014).

Thesis Outline

The honey bee has a sophisticated neuronal system capable of higher cognitive challenges like learning and memory and three dimensional navigation through a complex environment. Remodeling of the highly plastic brain occurs during an important age-related behavioral transition from inside-hive to outside tasks. The environment inside the dark, pheromone filled hive is drastically different from what the visually guided foragers experience in the enriched external world. Therefore, structural brain plasticity is likely to be an adaptation to new tasks in a changed environment. Accordingly, artificial light exposure is sufficient to evoke structural neuronal plasticity in visual brain centers, similar to effects found under natural conditions during the transition. Although environmentally induced brain plasticity occurs in animal species as diverse as humans and insects, the underlying molecular mechanisms are not fully understood. The honey bee, with its sequenced genome and advanced abilities for structural neuronal plasticity in a natural as well as in a confined artificial context, represents an excellent model organism to investigate mechanisms of experience-related neuronal plasticity. The two chapters of the present thesis address different aspects of the molecular underpinnings of this phenomenon in the honey bee.

Chapter I

Chapter I aims at linking light-induced structural neuronal plasticity with molecular changes in sub-compartments of the brain by asking following questions:

- Does light exposure influence the transcription of protein- and miRNA coding genes?
- Does age affect light-induced gene transcription?
- Is DNA methylation altered by light exposure?
- Are different sub-compartments of the brain subject to differences in light-induced molecular changes?

To address these questions, the transcriptome of age-matched light-exposed and dark-kept bees was compared. The experiment was performed for two age groups with two distinct brain sub-compartments. Furthermore, the DNA methylation pattern of one differentially expressed gene was examined for both treatments.

Results in Chapter I provide strong evidence for an implication of DNA methylation in structural neuronal plasticity. However, the relevance, dynamics, and mechanisms of DNA methylation are not well understood in the honey bee and shall be focused on in Chapter II.

Chapter II

To better comprehend the role of DNA methylation in structural neuronal plasticity, the second chapter of the thesis intends to further characterize two key regulators of DNA methylation, namely the DNA methyl transferase 3 (DNMT3), which catalyzes the *de-novo* methylation of cytosine to 5-methylcytosine (5mC), and TET which provides the conversion of 5mC to 5-hydroxymethylcytosine (5hmC). The following questions are raised:

- How are the expression of *DNMT3* and *TET*, and DNA methylation patterns, affected during the individual life history of the honey bee? Are the expression and methylation patterns rather age-dependent or correlated with task or experience?
- What is the sub-cellular localization of DNMT3?

To address these questions, gene expression levels of *DNMT3* and *TET* were quantified in a single cohort colony which provides age-matched nurses and foragers. Furthermore, the methylation patterns of two genes were compared between the individuals of the single cohort colony. The subcellular localization of DNMT3 protein was examined by immunohistochemistry in nurses and foragers.

A general introduction, giving a broad overview of relevant aspects, and a general discussion, bringing together the results of both chapters, build the framework of the thesis.

Chapter I

Age-dependent transcriptional and epigenomic responses to light exposure in the honey bee brain

Abstract

Light is a powerful environmental stimulus of special importance in social honey bees that undergo a behavioral transition from in-hive to outdoor foraging duties. Our previous work has shown that light exposure induces structural neuronal plasticity in the mushroom bodies (MBs), a brain center implicated in processing inputs from sensory modalities. Here we extended these analyses to the molecular level to unravel light-induced transcriptomic and epigenomic changes in the honey bee brain. We have compared gene expression in brain compartments of 1- and 7-day-old light-exposed honey bees with age-matched dark-kept individuals. We have found a number of differentially expressed genes (DEGs), both novel and conserved, including several genes with reported roles in neuronal plasticity. Most of the DEGs show age-related changes in the amplitude of light-induced expression and are likely to be both developmentally and environmentally regulated. Some of the DEGs are either known to be methylated or are implicated in epigenetic processes suggesting that responses to light exposure are at least partly regulated at the epigenome level. Consistent with this idea light alters the DNA methylation pattern of *bubblegum* (*bgm*), one of DEGs affected by light exposure, and the expression of miRNA miR-932. This confirms the usefulness of our approach to identify candidate genes for neuronal plasticity and provides evidence for the role of epigenetic processes in driving the molecular responses to visual stimulation.

This chapter is adapted from the manuscript:

Becker N, Kucharski R, Rössler W, Maleszka R. 2016. Age-dependent Transcriptional and Epigenomic Responses to Light Exposure in the Honey Bee Brain. *FEBS Open Bio*. accepted

The original article is available at:

http://onlinelibrary.wiley.com/doi/10.1002/2211-5463.12084/full

Introduction

Physiological and behavioral adaptations of an animal in response to novel experiences or to a changing environment are crucial for its fitness (Snell-Rood, 2013). One mechanism reflecting adaptation is neuronal plasticity, which is achieved via a complex interplay of environmental stimuli, intracellular signal transduction pathways and molecular mechanisms including DNA methylation, histone modifications and microRNAs (miRNAs) (Kolb and Whishaw, 1998; Borrelli et al., 2008; West and Greenberg, 2011; Maleszka, 2016). The interplay of these factors and their importance for adaptive behavior remains poorly understood.

Visual stimulation is one environmental factor that has been shown to induce neuronal plasticity in species as diverse as mammals and insects (Barth et al., 1997; Rybak and Meinertzhagen, 1997; Stieb et al., 2010; Kiorpes, 2015). One extensively studied example in this context comes from ocular dominance columns in the visual cortex of mammals, which respond preferentially to input from either one eye or the other. Monocular deprivation during a critical period shifts ocular dominance indicating the plasticity of this system upon environmental changes (Kiorpes, 2015). But even simple light exposure was shown to result in brain structural changes in Amphibia (Sin et al., 2002) and insects (Barth et al., 1997; Rybak and Meinertzhagen, 1997), including the honey bee (Hertel, 1983; Scholl et al., 2014). A number of studies have associated a few plasticity-related molecular processes and proteins with visually induced neuronal plasticity, for example, transcription of the immediate early genes *Arc* and *c-Fos* (Tagawa et al., 2005; Nakadate et al., 2013), recruitment of the cAMP pathway including PKA and CREB activity (Mower et al., 2002; Yuan et al., 2011), Nogo receptor 1 (Frantz et al., 2016), and Rho GTPases (Sin et al., 2002). However, the precise molecular mechanisms of light-induced neuronal plasticity and the interplay between different molecular pathways are still unclear.

The European honey bee, *Apis mellifera*, is a valuable model system to investigate this topic due to a sophisticated nervous system, rich behavioral repertoire and pronounced behavioral plasticity. With its sequenced genome and emerging epigenetic tools, the honey bee is becoming an organism of choice in studies aiming at unraveling the molecular mechanisms of environmentally induced neuronal changes underlying behavioral plasticity (Lyko and Maleszka, 2011).

Honey bee workers perform age-related tasks in the colony throughout their adult life (Lindauer, 1961). Young bees progress through a series of duties within the dark hive until after about three weeks of age they begin with foraging activity outside the hive which they commit to for their remaining life (Winston, 1991). A most important point during adult behavioral maturation is the switch from in-hive activities to outdoor foraging. This nurse-to-forager transition is associated with novel experiences in a rapidly changing environment. As foragers leave the dark pheromone-filled

hive and begin to search for food sources they become more visually-guided, particularly for localization of food sources and orientation using visual landmarks and sky-compass based navigation (Dyer, 1987). Therefore, foragers need to optimally adjust their visual system and behavior to novel environments and tasks and thus, adaptive changes in the nervous system of foragers have been described on the neuro-structural and molecular level.

The transition from nursing to foraging correlates with a volumetric increase of the MB (Withers et al., 1993), a prominent neuropil in the insect brain involved in sensory integration, memory formation and spatial orientation (Giurfa, 2007; Hourcade et al., 2010). The volume expansion depends on age and experience and is mainly caused by outgrowth of dendrites of the MB intrinsic neurons (Kenyon cells) (Farris et al., 2001; Maleszka et al., 2009; Muenz et al., 2015). At the same time, a density decrease (pruning) of synaptic complexes, so called microglomeruli (MG), takes place (Groh et al., 2012; Muenz et al., 2015). Most interestingly, exposing adult worker bees to light is sufficient to trigger MG pruning (Scholl et al., 2014). At the molecular level, high-throughput analyses of the nurse-to-forager transition have uncovered transcriptional changes of several hundred genes, some of which are known to modulate synaptic strength and synapse formation (Kucharski and Maleszka, 2002a; b; Whitfield, 2003; Whitfield et al., 2006; Lutz et al., 2012). This transition to foraging has also been associated with epigenetic changes at the level of DNA methylation and miRNA expression (Behura and Whitfield, 2010; Lockett et al., 2012). Altogether, these findings illustrate the high degree of neuro-structural- and molecular plasticity of the honey bee brain upon environmental changes which are partly driven by simple light exposure.

In this study, we have used the honey bee model to investigate environmentally induced brain plasticity at the level of transcription, DNA methylation and miRNA expression. In a broader context, our aim is to understand how sensory stimuli contribute to the genome-environment interplay that generates strikingly different phenotypes and behaviors without conventional genetic changes.

Materials and methods

An overview of our aims and experimental designs is shown in Table 1.

Table 1: Overview of the experimental design

Question	Experiment	Method	Treatment	Analysed brain regions	Analysed conditions & samples sizes	Results
Which protein coding genes are involved in light-induced neuronal plasticity in which brain regions?	1d-old light-exposed bees *vs*. 1d-old dark-kept bees	RNAseq of mRNAs	(i) Exposure to five light pulses (ii) Dark-kept control group	Optic lobes and central brain	1d L OL: 2 1d D OL: 2 1d L CBr: 2 1d D CBr: 2	See Table 3
Are results from RNAseq reproducible with qPCR?	1d-old light-exposed bees *vs*. 1d-old dark-kept bees	qPCR with 10 DEGs identified in RNAseq			1d L OL: 8 1d D OL: 8 1d L CBr: 8 1d D CBr: 8 7d L OL: 8 7d D OL: 8 7d L CBr: 8 7d D CBr: 8	See Table 4
Are DEGs in addition to light also regulated by age?	1d-old dark-kept bees *vs*. 7d-old dark-kept bees --- light-induced transcription in 1d-old bees *vs*. light-induced transcription in 7d-old bees					
Are miRNAs regulated by light and is this process involved in structural plasticity? **Does age influence miRNA expression?** **Are there differences between brain regions?**	1d-old light-exposed bees *vs*. 1d-old dark-kept bees --- 7d-old light-exposed bees *vs*. 7d-old dark-kept bees --- 1d-old dark-kept bees *vs*. 7d-old dark-kept bees	qPCR with 3 candidate miRNAs				See Table 5
Are DNA methylation patterns of DEGs altered by light?	7d-old light-exposed bees *vs*. 7d-old dark-kept bees	BS-MiSeq of the DEG *bgm*	(i) Exposure of 1d-old bees to five light pulses per day for 7 days (ii) Dark-kept control group		L OL: 1* D OL: 1* L CBr: 1* D CBr: 1*	See Figure 2

1d, 1-day-old bees; 7d, 7-day-old bees; L, light-exposed bees; D, dark-kept bees; OL, optic lobe; CBr, central brain; *Four replicates of the light experiment were performed. Eight brain structures (OLs or CBrs) were pooled per sample, whereby two structures derived from each of the four replicates (2 x 4 = 8).

Whole transcriptome sequencing

Animals

For whole transcriptome sequencing (RNAseq) newly emerged worker honey bees (*Apis mellifera ligustica*) were obtained from the Australian National University (ANU) apiary in Canberra. Two independent replicates of the following experiment were performed, one in April and one in May 2013. A comb with late pupae was taken from a hive, cleared of any bees, transferred to an incubator and kept at 34.5 °C in complete darkness. To collect age-matched bees, newly emerging individuals

were harvested within a 2 h time window under dim red light conditions. These young bees were transferred immediately as groups of 15 individuals to two wooden cages containing a small tube filled with honey from the same apiary. The caged bees were kept overnight in darkness at 32 ± 1 °C, 30-50% humidity.

Light exposure paradigm and sampling point

The next day, one cage of 1-day-old bees (~24h, referred as 1d) was exposed to five 45 min lasting pulses of artificial day light (light source: combined fluorescent tubes Repti-Glo 2.0 15W 45 cm and Repti-Glo 10.0 15W 45 cm from EXO-TERRA at 35 cm distance). Each light pulse was followed by a 75 min dark pause. This light protocol originates from a study with desert ants which aimed at simulating first exposure to light during first orientation (learning) walks (Stieb et al., 2010). In this species the protocol was shown to induce structural brain plasticity and with the same light program structural changes were also quantifiably in the honey bee brain after 3 days (Stieb et al., 2010; Scholl et al., 2014). Our intention in this study was not to mimic light exposure as occurs during first orientation flights of the honey bee, but solely to use this protocol as a tool to induce structural neuronal plasticity. The control cage remained in darkness. Directly after the fifth and last light pulse bees of the light and the dark group were immediately snap-frozen in liquid nitrogen and stored until further use at -80 °C. Bees in all experiments were sampled at the same time of day. We choose a sampling point on the first day of light exposure because we assumed that at this time point, a couple of hours after the initial light pulse, molecular processes mediating structural plasticity like transcription would be ongoing.

Library preparation

Frozen bees were partly thawed and brains quickly dissected in 50 mM NaCl, 25 mM Tris, 5 mM EDTA, pH 8 (0.5 x NTE buffer) as per our standard protocol (see a detailed video recording at https://db.tt/wSj9BBxL). The brains were split into optic lobes (OLs) and the rest referred to as central brain (CBr) and then transferred to separate 1.5 ml Eppendorf tubes kept on dry ice. Five CBrs or five pairs of OLs were pooled per sample. Samples were homogenized for 5-10 s with a plastic pestle (Sigma Z359947) attached to a hand-held motorized device. Total RNA was extracted using Trizol and then processed on magnetic beads (Dynabeads, Invitrogen) as per recommended protocol with the exception of the number of washes before final elution of mRNA that was increased to five. About 100 ng of rRNA-depleted mRNA was used for libraries construction with the NEBNext Ultra Directional RNA Library Prep Kit (#E7420S) and sequenced on the Illumina MiSeq machine (500 cycles kit MS-102-2023). Transcript variants level estimation- RNAseq reads from the GenBank SRA database were queried with 120 bp-long sequences covering symmetrically all predicted exon 4 3’splice junctions using standalone BLAST+. Specific junctions were identified and scored by analyzing

the resulting alignments; a score was incremented if there was a continuous (ungapped) alignment of minimum 70 nucleotides. Transcript content is estimated as a percentage of a specific junction in all junctions analyzed. *Apis mellifera* genome assembly v.4.5 was used (www.beebase.org). RNAseq data are available at http://dna.anu.edu.au. Libraries were prepared for each treatment group (light, dark) and brain region (OL, CBr) from two independent biological replicates of the experiment, resulting in a sample size of 2 for each condition (light OL, light CBr, dark OL, dark CBr).

Quantitative real-time PCR

Animals

For quantitative real-time PCR (qPCR), worker honey bees (*Apis mellifera* var. *carnica*) were obtained from colonies of the apiary at the Biocenter, University of Würzburg, Germany from July to October 2013, and in August 2014. Bee collection and bee handling were performed as described above for the RNAseq experiments with the exception of feeding which was with 50 % Apiinvert (Südzucker), and the time window for collecting newly emerged bees, which was extended to 8 hours.

Light program and sampling point

The light protocol for 1- and 7-day-old bees was the same as for RNAseq. For qPCR experiments with 7-day (7d) old bees, the newly emerged bees were kept for six days at 32 ± 1°C, 30-50% humidity in cages in total darkness before starting the light treatment on the seventh day after eclosion. Sampling again took place directly after the fifth light pulse for both age groups.

Sample preparation

Primers (Table 2) for qPCR experiments were designed on the basis of the *Apis mellifera* Genome Assembly 4.5. Their specificity could be validated by a BLAST search against the *Apis mellifera* genome, by gel electrophoretic analysis of the PCR products and by a melt curve analysis. Their efficiency (E) was determined in a standard curve analysis by the Eppendorf Mastercycler ep *realplex* software version 2.2.0.84 (Eppendorf) with a non-diluted and diluted (1:2, 1:4, 1:8) samples (Table 2). The forward primers for the miRNAs were designed on basis of the sequences available at mirBase (www.mirbase.org/). The forward primer for the non-coding reference RNA *RNU6-2* (*GB50324*) and reverse primers for miRNA quantification were obtained from the miScript II RT Kit (Qiagen). Note that the provided *RNU6-2* primer assay was designed against the human sequence (Entrez Gene ID: 26826). The integrity of this primer assay for use in *Apis mellifera* could be validated by a blast search with the human *RNU6-2* sequence against the *Apis mellifera* genome, by a gel electrophoretic analysis with the PCR product of the primer assay, and melt- and standard curve analyses.

Brain dissections were performed as mentioned for RNAseq. The OLs and the CBrs from three brains were pooled, respectively. The sample size for each tested gene is indicated in Table 4. RNA was

extracted by homogenizing the tissue with a 5mm steel bead (Qiagen) in 500 µl Trizol on a Tissue Lyser LT (Qiagen) for 3 min at 40 Hz. Subsequent RNA extraction steps were conducted according to the Trizol manufacturers' guide. The RNA pellet was re-suspended in 20 µl RNAase-free water by heating the sample at 80 °C for 2 min. RNA concentration and purity was measured with a µCuvette G1.0 (Eppendorf) in a BioPhotometer plus (Eppendorf). RNA integrity was determined for a few samples by gel electrophoretic analysis.

cDNA was synthesized from mRNA with the QuantiTect Rev. Transcription Kit (Qiagen) according to the manufacturers guide. One microgram total RNA was used as starting material. In the final step the cDNA was diluted 1:10 by adding 180 µl TE-buffer (10 mM Tris, 1 mM EDTA, pH 8). cDNA for miRNA analysis was synthesized with the miScript II RT Kit (Qiagen) according to the manufacturers guide. One microgram total RNA as starting material and the miScript HiFlex Buffer were used. The resulting cDNA was diluted 1:5 by adding 80 µl TE-buffer.

For relative quantification of mRNA levels via qPCR, 2 µl of the respective diluted template cDNA was mixed with 10 µl KAPA SYBR FAST qPCR MasterMix (peqlab), 200 nM of the forward- and reverse primer each, and RNAse-free water to fill up to a final volume of 20 µl. qPCR was run on an Eppendorf Mastercycler ep gradient s realplex² (Eppendorf) with following program settings: 5 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, and extension at 72 °C for 30 s. Melt curves were accessed with the following program: 95 °C for 15 s, followed by rapid cooling to 60 °C and then heating to 95 °C in increments in 20 min. *RpL32* (former *rp49*, GB47227) was used as reference gene in each qPCR run (Lourenco et al., 2008; Reim et al., 2012). Each sample was analyzed in technical triplicates. Ct-values were determined with the default settings by the Cycler's software (Eppendorf Mastercycler ep *realplex*). For relative quantification of miRNA levels via qPCR, 2 µl of the respective diluted template cDNA was mixed with 10 µl KAPA SYBR FAST qPCR MasterMix, 500 nM of the forward primer, 500 nM of the reverse miScript Universal Primer (Qiagen), and water to fill up to a final volume of 20 µl. The same qPCR program was used as described above, except for the annealing temperature, which was at 55 °C. *RNU6-2* (GB50324) served as a reference non-coding RNA.

To determine whether two groups show a statistically significant difference in the expression level of a respective gene, first the normalized ct-values ($ct_{norm,\ tar}$) of the respective target gene from each sample was calculated by subtracting the ct-value of the reference gene (ct_{ref}) from the ct-value of the target gene (ct_{tar}): $ct_{norm,\ tar} = ct_{tar} - ct_{ref}$. Second, the normalized ct-values of the target gene from each replicate of one test group were compared to the normalized ct-values of the target gene of a second group via an independent t-test with the statistics program IBM®SPSS®Statistics 21.

The relative expression ratios (*R*) and standard errors were calculated with the Pfaffl-method (Pfaffl, 2001; Pfaffl et al., 2002).

Samples were prepared for each treatment group (light, dark) and brain region (OL, CBr) from eight independent biological replicates of the experiment, resulting in a sample size of eight for each condition (light OL, light CBr, dark OL, dark CBr) and gene as stated in Table 4.

Table 2: Primer sequences for qPCR and nested PCR

Symbol	Full name	BeeBase gene ID	Forward-/reverse primer	Primer efficiency
GB41720	*uncharacterized LOC727121*	GB41720	CGACCAACACCATGCTACCT/ CGTAACATTCGAACGGCGAC	1,91
GB48020	*uncharacterized LOC552041*	GB48020	ACGAAGCGATACAACTTACGGT CGTATTGCTCTATTCAGTGCGTC	1,9
GB55613	*uncharacterized LOC100576118*	GB55613	CTGAACGCGACAGAAACGAC/ TCTGATTGGTTCAGAGCGTCA	1,98
Ip3ka	*inositol 1,4,5-triphosphate kinase 1*	GB41220	GCCGGCCAGTGACGTATTAT/ TTCCACTTCTCTGTAATATCTTGGT	1,93
Jhbp-1	*take-out-like carrier protein (juvenile hormone binding protein-1)*	GB48492	ACCCAATACACATAGACTGGGA/ GCAGGATTGAATTTCACCGCA	2,35
L(2)efl	*protein lethal(2)essential for life*	GB45913	ACCTTGGGGTGAACTTCTGC/ CCCTCGACGACAACACACTT	1,92
RpL32	*ribosomal protein L32*	GB47227	CGTCATATGTTGCCAACTGGT/ TTGAGCACGTTCAACAATGG	2,07
Tim2	*timeout*	GB41002	TGCAAGTGCTAGACATTCCCAT/ GGACGTTTGTTTTTCGGTTTCG	1,99
Trim71	*tripartite motif-containing protein 71*	GB48462	TCGTATCCAGGTGTTGACGAT/ ACGATGTTGCCGTCAGGATT	1,99
Uty	*histone demethylase UTY*	GB54595	GTCAACGCATCCAGGGGTAA/ GGTGCTTGGCTCAGATGACT	1,97
miR-210	*miR-210*	MI0001581 (miRBase.org)	TTGTGCGTGTGACAGCGGCTA/ miScript Universal Primer (Qiagen)	2,08
mir-932	*miR-932*	MI0005754 (miRBase.org)	TCAATTCCGTAGTGCATTGCAG/ miScript Universal Primer (Qiagen)	2,04
miR let-7	*miR let-7*	MI0005726 (miRBase.org)	TGAGGTAGTAGGTTGTATAGT/ miScript Universal Primer (Qiagen)	2,01
RNU6-2	*uncharacterized LOC724988*	GB50324	*RNU6-2* miScript Primer Assay (Qiagen)	2,01
bgm	*very long-chain-fatty-acid--CoA ligase bubblegum*	GB51580	outer primers: TTTTTTAATAATTTTAGGTAGTTG/ AATAAATACTTACTTCAAATTTAC nested primers: GCAGAATTC-TATTTTATGTTATATATAGTTGGT/ CGCAAGCTT-CTAATATATTCACAATATATACAC	/

Bisulfite sequencing with MiSeq

Animals

Bees used for bisulfite sequencing with MiSeq (BS-MiSeq) were obtained from colonies of the apiary at the Biocenter, University of Würzburg in August 2014.

Light exposure paradigm and sampling point

Newly emerged bees were transferred to cages and exposed for 7 days, instead of the usual 1 day, to light pulses. After the fifth light pulse of each day the bees remained in the dark overnight as

described in (Scholl et al., 2014). An age-matched control group was kept in the dark. Bees were sampled after the last pulse of the seventh day. As nothing is known about the dynamics of DNA methylation in the honey bee, we decided to extend the light program to 7 days to ensure enough time for the establishment of quantifiable changes in the DNA methylation pattern.

Library preparation

Bisulfite sequencing was performed as previously described (Kucharski et al., 2015; Wedd et al., 2016) with the following adjustments. For each treatment group (light, dark), 8 MBs and 8 pairs of OLs, respectively, were pooled. The brains for this experiment derived from four independent biological replicates of the experiment, whereby two brains from each replicate were included in the pool. DNA from the four pools (light OL, light MB, dark OL, dark MB) was extracted with the NucleoSpin® Tissue XS kit from Machery-Nagel according to the manufacturers' protocol. Two microgram DNA was used for initial bisulfite conversion. Nested PCR was conducted with primers indicated in Table 2 which flank four CpGs in *bubblegum* (*bgm*). For library preparation 250 ng of amplicons for each tested group were applied to the NEBNext® DNA Library Prep Master Mix for Illumina®, and NEBNext® Multiplex Oligos for Illumina® Index Primers Set 1-4 were used for the different samples.

Prediction of putative target genes of *miR-932*

Targets of *miR-932* were bioinformatically predicted as previously described in (Ashby et al., 2016).

Phototaxis assay

Newly emerged bees from the apiary at the Biocenter, University of Würzburg were collected in September 2015, separated into four groups, and transferred to cages and exposed to the same light protocol as for the molecular studies. The four groups were (a) bees exposed to light pulses on the first day after eclosion (1d light), (b) an age-matched dark-kept control group (1d dark), (c) bees kept in a dark incubator for 6 days before exposure to light pulses on the seventh day after eclosion (7d light), and (d) an age-matched dark-kept control group (7d dark). Bees were tested for phototaxis on the day after light treatment to provide a close temporal frame to the molecular studies which may allow an interpretation of potentially altered phototaxis by light-induced molecular changes.

Phototaxis was tested in an arena described previously (Erber et al., 2006; Thamm et al., 2010). In short, the arena is a lightproof circular construction with 28 cm diameter. Green light emitting LEDs of different relative intensities (12.5 %, 25 %, 50 %, 100 %) were installed in the walls with two LEDs of the same intensity positioned opposite to each other. Movements of the bee were recorded via an infrared camera. The bees were put in the dark arena and given two minutes to adapt. Then the lowest intensity LED was switched on. Whenever the bee reached the LED it was turned off and the

opposite LED of the same intensity was switched on. This procedure was repeated four times for each intensity. A bee moving between the two LEDs in a directed manner in at least one of the four trials for the respective light intensity was counted as positive phototaxis for that intensity. Significance was calculated with the Chi-squared test in IBM®SPSS®Statistics 21.

Results

Light affects the transcription of protein-coding candidate genes for neuronal plasticity

For a hypothesis free approach of finding genes with transcriptional changes affected by light exposure we performed two independent RNAseq experiments using mRNAs extracted from the OLs and the CBr of 1-day-old bees exposed to light and kept in darkness. Although a few hundred genes have shown a detectable level of transcriptional change, many differences became very small after combing the two RNAseq datasets and such genes were not counted as differentially expressed. Only genes with around 2-fold change in the same direction in both RNAseq datasets were considered further to lower the risk of reporting false positive hits. In experiment 2, a few genes show a very high induction (indicated as 100) suggesting that precise timing is one factor affecting the level of light-inducible transcripts. This approach has identified 52 genes between the two treatment groups (Table 3). The list of DEGs contains genes belonging to a few functional categories: (i) neuronal plasticity (*bgm* (Min, 1999)*, Cnpy-1* (Bornhauser, 2003), *Ip3ka* (Windhorst et al., 2012)), (ii) epigenetic functions (histone demethylase *Uty* (Agger et al., 2007), *histones H3 and H4* (Szenker et al., 2011), and *Trim71* (Zou et al., 2013)*),* (iii) metabolism/energy flux (*GB42985 – n-acetylneuraminate lyase* (Sanchez-Carron et al., 2011)*, GB45023 - alpha-tocopherol transfer protein* (Lim and Traber, 2007)*, GB55050 - solute carrier family 26 member 6* (Chernova et al., 2005)), and (iv) signal transduction (*GB55043 - glutamate receptor, ionotropic kainate 2* (Ozawa et al., 1998)). A relatively large proportion of DEGs (9 out of 52 (17%)) falls into the fifth unknown/novel category. 11 out of 52 DEGs have been shown to be methylated and are predicted to be regulated at the epigenome level.

Of special interest for our study are genes listed in the first functional category (i) because of their direct implication in neuronal plasticity. For example, *Ip3ka* encodes a protein that accumulates in dendritic spines in the hippocampus after long-term potentiation in mice and after spatial learning tasks in rats (Kim et al., 2004, 2009). *Ip3ka* knock-out mice show a decrease of dendritic-spine density in the dentate gyrus and defects in memory performance (Kim et al., 2009). Furthermore, it is proposed that *Ip3ka* modulates dendritic structures by its interaction with f-actin (Windhorst et al., 2012). *Cnpy-1* may also contribute to structural plasticity in the honey bee brain as the overexpression of this gene leads to neurite outgrowth in cell cultures (Bornhauser, 2003). Finally, *bgm* is important for the correct formation of the OLs in adult flies and is suggested to play a role in myelinogenesis (Min, 1999; Steinberg et al., 2000).

In the OLs, all DEGs except *Uty* show upregulation after light induction suggesting that light exposure tends to activate transcription of most genes in the optic lobes. The role of *Uty* gene in the honey bee is not known, but K27 methyl mark on histone H3-K27 is part of transcriptional regulation in

mammals. Therefore, it is likely that in our experiment, light- influenced responses of *Uty* also imply similar regulatory function (Agger et al., 2007).

We chose seven of the 52 DEGs in the OLs for additional qPCR analyses using material derived from independent replicates of the experiment. Of these seven genes, five (*Cnpy-1, GB55613, Ip3ka, Tim2,* and *Trim71*) show the same direction of differential expression as found with RNAseq, whereas two genes (*Uty, Jhbp-1*) show an opposite direction (Table 4). Of the consistent five genes, three (*Cnpy-1, Ip3ka, Trim71*) show a statistically significant differential expression between the two treatment groups. We also have tested these seven genes for differential expression between the treatment groups in the OLs of older bees (7-days of age) with qPCR. Again, *Cnpy-1, Ip3ka,* and *Trim71* show a statistically significant difference between the light- and the dark group (see Table 4) in the qPCR study.

In contrast to the OLs, in the CBr of 1-day-old bees RNAseq has revealed a much lower number of only eight DEGs (Table 3). From this list, one gene (*L(2)efl*) was reported to have a direct function in neuronal plasticity. *L(2)efl* is linked to Charcot-Marie-Tooth neuropathy (Evgrafov et al., 2004), and known to mediate neurite growth in sensory neurons (Williams and Mearow, 2011). This may be due to its interaction with the cytoskeleton, especially with f-actin (Wettstein et al., 2012).

From the eight DEGs in the CBr, three (*GB41720*, *GB48020*, *L(2)efl*) were tested with qPCR with material from independent replicates of the experiment. *GB41720* and *L(2)efl* show a tendency towards a higher expression in the light group, which was in line with the results from RNAseq (Table 4). The differential expression of *L(2)efl* is close to a significant *P*-value (independent *t*-test: *P*-valueb = 0.054). *GB48020* shows an opposite direction of expression as seen with RNAseq. Next, we have examined the same three genes for differential expression between the light and dark group in the CBr of 7-day-old bees via qPCR. *L(2)efl* shows a statistically significant 2.01 fold higher expression in the light group (Table 4). *GB41720* tends to be slightly up-regulated (1.12 fold) in the light group as well, but a significance level was not reached (independent *t*-test: *P*-value = 0.130). No significant differential expression is seen for *GB48020.*

Altogether, 70 % (7 out of 10) of the DEGs identified via RNAseq and tested with qPCR show the same tendency of change in both methods, which confirms the robustness of our assay. Possible reasons for the 30 % discrepancy may have resulted from experimental differences between the two methods. Bees for RNAseq derived from Canberra (Australia), belong to the races *ligustica,* and were fed with honey, whereas bees for qPCR came from Würzburg (Germany), are *carnica* and fed with a sugar solution. Therefore, it seems likely that the divergence may be explained by a differential behavioral or physiological state of the two groups of bees.

Table 3: Light-induced DEGs in the OLs and CBr identified with RNAseq

Gene ID	*R* (Log2 ratio) Experiment 1	*R* (Log2 ratio) Experiment 2	Methylated	General function
Optic lobes				
***GB55613**	6.10	100.00	Yes	Unknown
***Uty* (GB54595)**	-1.22	-1.29	Yes	Histone H3K27 demethylase
GB45148	1.14	1.77		Vitamin A related
GB45147	1.28	3.13	Yes	Vitamin A related
GB45024	0.69	1.00		Vitamin A related
GB45023	0.57	2.66		Vitamin A related
***Ip3ka* (GB41220)**	2.30	1.20	Yes	IP3 kinase
GB42985	3.53	1.96		Pyruvate lyase
***Tim2* (GB41002)**	2.32	1.47		Timeless
GB43805	1.20	1.80		Metallo-endopeptidase
GB46312	2.86	2.37		Cuticular protein
GB55396	1.28	3.16		Unknown
***Cnpy-1* (GB50831)**	2.02	2.45	Yes	Neurite outgrowth enhancer
***Trim71* (GB48462)**	1.30	1.42		E3 ubiquitin protein ligase
GB43732	1.13	1.80		Serine/threonine-proteinkinase
GB44871	2.38	2.36		GglycineN-methyltransferase
GB47279	3.50	3.60		Cytochrome P450
GB43514	3.04	100.00		Lipase, memberH
GB49843	3.39	2.79		Neuronal PAS domain protein
GB54962	1.12	4.19		Unknown
GB42197	3.73	1.09		Unknown
***Histone H3* (GB47484)**	1.41	1.68		Histone H3
GB47382	1.31	3.19		HistoneH4
GB41720	1.98	2.74		Pleckstrin
***Jhbp-1* (GB48492)**	1.07	1.32	Yes	Take-out
GB42467	2.91	7.10		Phototransduction
GB42673	1.54	2.16		RDH10/retinoldehydrogenase
GB43649	1.31	1.17		Chloride channel
GB55043	2.57	1.87		Kainate glutamate receptor
GB43823	2.83	4.72	Yes	Chemosensory protein CSP1
GB41593	3.22	3.28	Yes	Cell migration regulator
GB40046	1.43	100.00		Neuronal mt transport protein
GB55050	100.00	100.00		Transmembrane transporter
GB41277	1.14	3.29	Yes	light-induced ubiquitylation
GB45365	1.08	1.88		Transmembrane transporter
GB47948	1.47	3.08		Myosin light chain kinase
GB41720	1.98	2.74		Plekstrin
GB51220	1.20	1.32		Cytochrome b-561
GB40552	2.69	3.02		Unknown
GB45910	1.23	1.31		Crystallin
GB45906	1.05	1.07		Crystallin2
GB46514/ GB46515	1.19	1.46	Yes	Acetylcholinesterase (bothloci)
GB44095	1.60	3.11		Cation channel
GB42227	4.30	3.59		Homeobox related
***bgm* (GB51580)**	1.91	1.73	Yes	Acyl-CoA synthetase
GB41339	2.22	100.00		Acid phosphatase
GB52448	2.75	2.53		Unknown
GB53210	2.22	2.57		Unknown
GB47697	1.79	1.04		Unknown
GB41709	2.20	1.21		Unknown
Central brain				
***GB41720**	1.52	1.00		Low density lipoprotein receptor adapter
***GB48020**	-1.04	-0.76		flocculation protein FLO11
***L(2)efl* (GB45913)**	1.26	1.51		protein lethal(2)essential for life
GB44549	-1.43	-1.59		glucose oxidase
GB41310	2.69	1.30		Actin
GB45796	-2.96	-1.26		Major royal jelly protein 3
GB41309	1.92	2.58		Unknown
GB41307	1.90	2.43		Unknown

R, relative expression ratio (Log2) of gene expression levels in the light group compared to the dark group; 100.00, Because there is virtually no expression in one condition the increase is shown as 100; *Genes checked with qPCR.

Table 4: Effect of light exposure and age on the transcription of protein coding genes in the OLs and CBr determined by qPCR

Symbol	Light vs Dark						7-day-old vs 1-day-old					
	1d light / 1d dark			7d light / 7d dark			7d dark / 1d dark			7d light / 1d light		
	R (Log2)	*n*	*P*-value	*R* (Log2)	*n*	*P*-value	*R* (Log2)	*n*	*P*-value	*R* (Log2)	*n*	*P*-value
Optic lobes												
Cnpy-1	0.51	8	**	0.80	8	***	0.42	8	**	0.72	8	***
GB55613	0.71	8	n.s.	0.69	8	n.s.	0.32	8	n.s.	0.30	8	n.s.
Ip3ka	0.90	8	***	1.02	8	***	-0.62	8	**	-0.51	8	**
Uty	0.04	8	n.s.	0.55	8	n.s.	-1.36	8	**	-0.84	8	*
Jhbp-1	-0.15	4	n.s.	0.23	4	n.s.	-4.06	4	**	-3.64	4	**
Tim2	0.07	8	n.s.	-0.22	8	n.s.	-0.94	8	0.054	-1.22	8	*
Trim71	1.07	8	***	2.29	8	***	-0.58	8	0.050	0.65	8	**
Central brain												
GB41720	0.15	8	n.s.	0.16	8	0.130	-0.38	8	*	-0.36	8	*
GB48020	0.38	8	n.s.	-0.09	8	n.s.	-1.06	8	n.s.	-1.51	8	**
L(2)efl	0.19	8	0.054	1.01	8	*	0.62	8	*	1.43	8	**

R, relative expression ratio (Log2); *n*, samples size for each group; 1d, 1-day-old bees; 7d, 7-day-old bees; *P*-value, independent *t*-test comparing normalized ct-values of the two respective groups; **P*-value < 0.05; ***P*-values < 0.01; ****P*-value < 0.001; n.s., *P*-value ≥ 0.05.

Age affects the transcription of protein-coding candidate genes for neuronal plasticity

Comparison of the candidate gene expression levels in the OLs between the two age groups (1- and 7-day-old bees) by qPCR reveals age-related differences. Four out of seven tested candidate genes are expressed significantly different between the 7d dark group and the 1d dark group (*Cnpy-1, Ip3ka, Uty, Jhbp-1*), and two genes (*Tim2* and *Trim71*) show a strong tendency towards differential expression (Table 4). *Ip3ka, Uty, Jhbp-1*, *Trim71* and *Tim2* are expressed at lower levels in 7-day-old bees with only *Cnpy-1* showing higher expression.

In the CBr, age-related differences in candidate gene expression also are apparent for the three tested genes *GB41720*, *L(2)efl* and *GB48020*. Comparing the expression levels of the 1- and 7-day-old dark-kept bees reveals a significantly lower expression of *GB41720* and a strong, but non-significant, trend towards a lower expression of *GB48020* in the 7-day-old group (Table 4). In contrast, the expression of *L(2)efl* in 7-day compared to 1-day-old bees is significantly higher.

Most interestingly, age appears to affect the amplitudes of light-induced gene transcription. The light-induced expression of *Cnpy-1, Ip3ka, Trim71* and *L(2)efl* is more pronounced in 7-day-old bees (Table 4). This is particularly obvious for *Trim71* with 2.33 times higher levels of light-induced expression in the OLs of 7-day compared to 1-day-old bees ($R_{7d\ light/7d\ dark}/R_{1d\ light/1d\ dark}$).

Furthermore, age also seems to influence the standard deviation of gene expression which was higher for seven out of ten genes in 7-day-old bees compared to 1-day-old bees (Fig. 1).

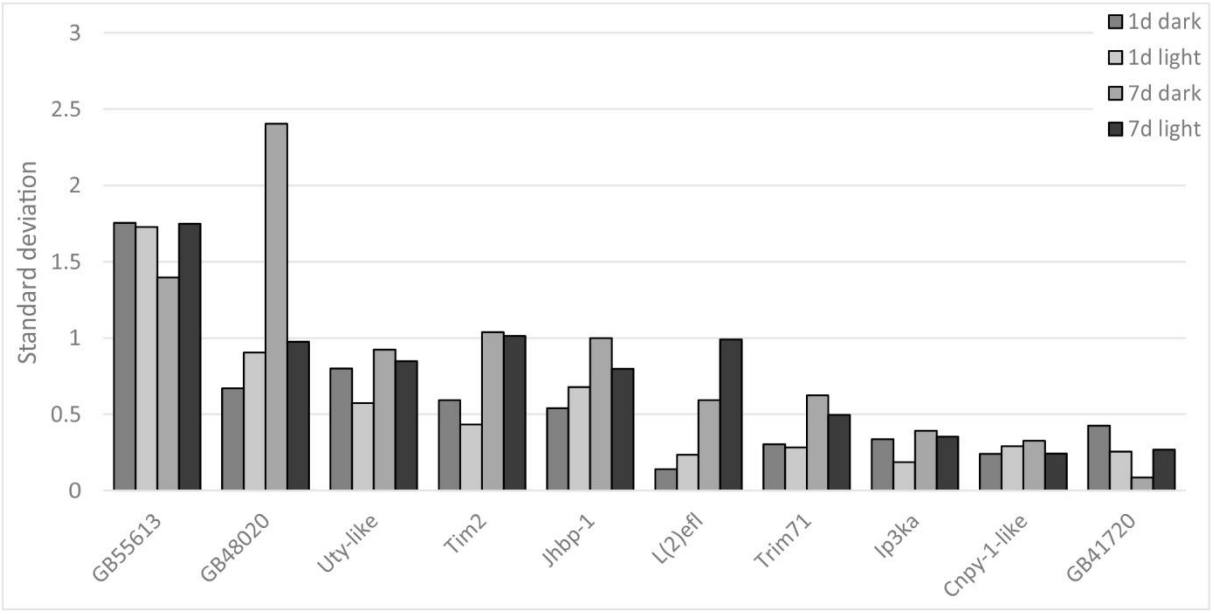


Figure 1: Standard deviation of the expression of DEGs. The standard deviation for the expression of seven out of ten DEGs is higher in 7-day-old bees compared to 1-day-old bees. 1d, 1-day-old bees; 7d, 7-day-old bees; dark, dark-kept bees; light, light-exposed bees.

Light-, age- and brain-compartment related expression of candidate microRNAs

Since in the OLs both light exposure and age appear to strongly influence the expression of *Trim71*, a known target of the miRNA *let-7* (*miR let-7*) (Lin et al., 2007), we asked whether the expression levels of this miRNA correlate with *Trim71* levels. Our qPCR analysis has not revealed any light-inducible effects on *miR let-7* in both the OLs and CBr of 1- and 7-day-old light-exposed and dark-kept bees (Table 5). However, age strongly affects the expression levels. In the OLs of 7-day-old bees the *miR let-7* level was half as low as in 1-day-old bees (0.47 fold), which correlates with more than twice as high (2.33) light-induced *Trim71* expression in 7-day compared to 1-day-old bees. In other words, low *miR let-7* levels correlate, in an age-dependent manner, with relatively high light-induced *Trim71* levels and vice versa. Interestingly, a similar age-dependent correlation was described for *C. elegans,* in which age-dependent expression of *miR let-7* differentially regulates axon growth potential through its interaction with *lin-41* (the homolog of *Trim71*). High levels of *miR let-7* in old neurons inhibit *lin-41* expression leading to a decline in axon plasticity, whereas in young neurons low levels of *miR let-7* result in unhampered *lin-41* expression maintaining axon plasticity (Zou et al., 2013). Therefore, in the honey bee brain age-dependent *miR let-7* levels may be a critical factor determining the extent or onset of environmentally induced neuronal plasticity mediated by *Trim71*.

We have quantified the expression levels of two further miRNAs in the OLs and the CBr of 1- and 7-day-old light-exposed and dark-kept bees, *miR-923* and *miR-210*, which have been linked to brain functions in the honey bee (Cristino et al., 2014; Qin et al., 2014; Ashby et al., 2016). The expression of *miR-932, but not miR-210,* shows a significant light effect (Table 5). In the OLs, the expression of *miR-932* is 1.12 fold higher in 1-day-old light-exposed bees compared to age-matched dark-kept ones

(independent *t*-test: *P*-value = 0.036). This light effect persists in the OLs of 7-day-old bees, but with no statistical significance (independent *t*-test: *P*-value = 0.107). As *miR-932* shows a transcriptional response to light, we predicted its putative targets (Table 6). These include *GB44947* and *GB45281* which have reported functions in neuronal plasticity in other organisms. *GB44947* homologs (*Doublecortin*) are involved in proper f-actin formation, microtubule stabilization, and neuronal migration (Fu et al., 2013). The homolog of *GB45281* (*E3 ubiquitin-protein ligase Hyperplastic discs*) regulates *hedgehog* and controls photoreceptor differentiation in *Drosophila* and, therefore, is a good candidate for adaptation processes in the honey bee eye in response to sensory stimuli (Lee, 2002). Although no differential expression of *GB44947*0 or *GB45281* has been detected in our study, it is conceivable that *miR-932* affects their regulations at specific time points after light exposure. Furthermore, miRNAs have the ability to subtly fine-tune gene transcription at distinct subcellular locations (i.e. at synapses or even dendrites) which would be unlikely to detect with our approach extracting total RNA from entire brain areas (Aksoy-Aksel et al., 2014).

Table 5: Effect of light exposure, age, and brain compartment on microRNA expression determined by qPCR

Light vs Dark												
Symbol	**OLs**						**CBr**					
	1d light / 1d dark			**7d light / 7d lark**			**1d light / 1d dark**			**7d light / 7d dark**		
	***R* (Log2)**	***n***	***P*-value**	***R* (Log2)**	***n***	***P*-value**	***R* (Log2)**	***n***	***P*-value**	***R* (Log2)**	***n***	***P*-value**
miR let-7	0.01	8	n.s.	0.07	8	n.s.	0.01	8	n.s.	-0.01	8	n.s.
miR-210	0.06	8	n.s.	-0.06	8	n.s.	0.08	8	n.s.	-0.04	8	n.s.
miR-932	0.16	8	*	0.10	8	n.s.	0.07	8	n.s.	-0.06	8	n.s.
7-day-old vs 1-day-old												
	OLs						**CBr**					
	7d dark / 1d dark			**7d light / 1d light**			**7d dark / 1d dark**			**7d light / 1d light**		
miR let-7	-1.09	8	***	-1.03	8	***	-0.47	8	***	-0.49	8	***
miR-210	-0.27	8	*	-0.36	8	**	-0.42	8	***	-0.56	8	***
miR-932	0.01	8	n.s.	-0.06	8	n.s.	0.21	8	**	0.08	8	0.074
Optic lobes vs Central brain												
	Age: 1d						**Age: 7d**					
	dark OL / dark CBr			**light OL / light CBr**			**dark OL / dark CBr**			**light OL / light CBr**		
miR let-7	0.03	8	n.s.	0.03	8	n.s.	-0.58	8	**	-0.49	8	**
miR-210	-0.43	8	**	-0.47	8	**	-0.27	8	**	-0.29	8	**
miR-932	-0.40	8	***	-0.30	8	***	-0.60	8	***	-0.43	8	***

R, relative expression ratio (Log2); *n*, samples size for each group; 1d, 1-day-old bees; 7d, 7-day-old bees; OL, optic lobes; CBr, central brain; *P*-value, independent *t*-test comparing normalized ct-values of the two respective groups; **P*-value < 0.05; **, *P*-values < 0.01; ***P*-value < 0.001; n.s., *P*-value ≥ 0.05.

Table 6: Putative targets of *miR-932*

Honey bee			Fly ortholog	
Gene ID	**Symbol**	**General function**	**Symbol**	**General function**
GB50397	/	unknown	PDZ-GEF	PDZ domain-containing guanine nucleotide exchange factor
GB44947	LOC726454	similar to CG13467-PA	DCX-EMAP	Doublecortin-domain-containing echinoderm-microtubule-associated protein
GB44221	Noc2	nucleolar complex protein 2	CG9246	
GB54520	/	unknown	/	/
GB47477	LOC726348	similar to peroxisomal biogenesis factor 6	Pex6	Peroxin 6
GB54355	yps	ypsilon schachtel	yps	ypsilon schachtel
GB55860	/	unknown	Ect4	Ectoderm-expressed 4
GB55364	/	unknown	Ptp99A	Protein tyrosine phosphatase 99A
GB45281	hyd	E3 ubiquitin-protein ligase hyd	hyd	hyperplastic discs
GB41610	/	unknown	/	/
GB44526	LOC551919	similar to Paxillin CG31794-PC, isoform C	Pax	Paxillin

Light affects DNA methylation of *bubblegum*

11 DEGs from our study are known to be methylated. To examine if DNA methylation changes are associated with light exposure in these DEGs, we have used ultra-deep bisulfite sequencing of gene-specific amplicons (Kucharski et al., 2015; Wedd et al., 2016). This method has the capacity to generate up to one million reads per each amplicon and its resolving power is sufficient to visualize all condition-specific methylation patterns that may be associated with dozens of distinct cell-types even if methylation levels in certain cell types are very low. We selected one of the DEGs, *bgm*, as the illustrator gene because in previous analyses it has shown a relatively high level of methylation in a short region of DNA spanning four CpG sites (see Table 2 for primers flanking this genomic region). The protein encoded by *bgm* plays a central role in brain long-chain fatty acids metabolism and myelinogenesis, and in correct development of the OLs in adult flies (Min, 1999; Steinberg et al., 2000). It also has a role in global epigenetic control of transcription because it supplies acetyl-CoA for histone acetylation by histone acetyltransferases (Takahashi et al., 2006). As shown in Figure 2, *bgm* methylation patterns are responsive to light exposure, especially in the OLs where there is more than 11 % more methylation seen at all four CpGs in a certain proportion of patterns, but with CpGs #2 and 4 most affected. The light influence also is detectable in the MBs, but the increase in methylation in this neuropil is less pronounced (5.14 %). Given the very high sequencing coverage in each sample, it is likely that patterns showing the highest methylation dynamics represent a few specific cell types that are primarily responsible for processing light signals in both brain compartments.

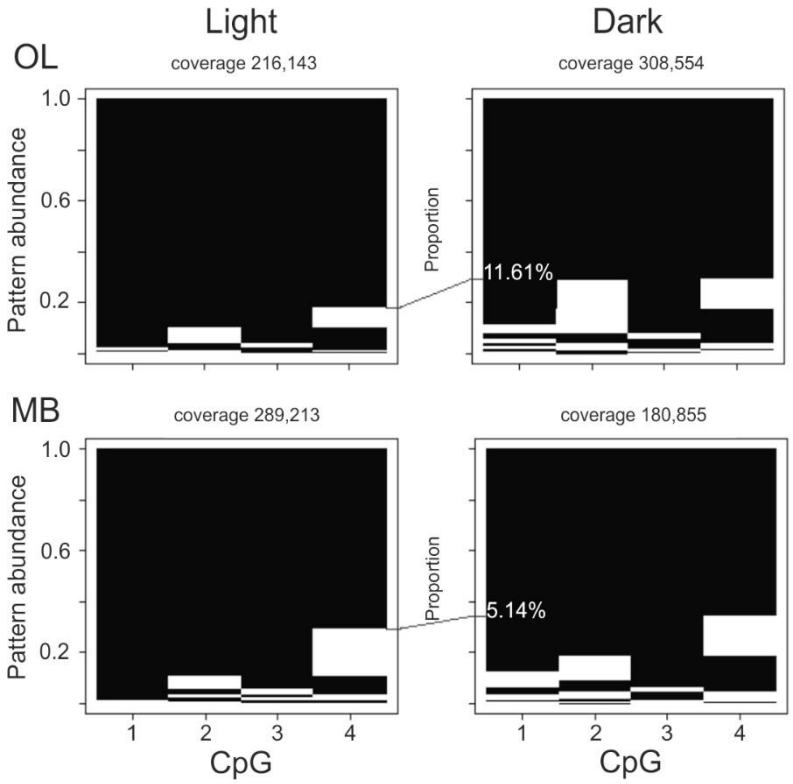


Figure 2: Effect of light on the methylation pattern of *bgm*. Methylation patterns in *bgm* revealed by deep amplicon sequencing. Each row represents a methylation pattern (black: methylated CpGs, white: not methylated CpGs), the height of each pattern is proportional to the pattern's abundance. *bgm* amplicons were amplified from both optic lobes and MBs using light-exposed and dark-kept bees. After normalizing pattern frequencies several distinct and highly abundant methylation patterns have been detected. The pattern proportions are sorted from the most abundant at the top to the least abundant at the bottom. The number of sequenced reads for each situation is shown above each panel. OL, optic lobes; MB, mushroom bodies.

Age and possibly light treatment affect phototaxis

Given the effect of light exposure on the transcription of several genes in an age-dependent manner, we were interested whether light treatment and age have an effect on vision-related behavior, in particular phototaxis. We found that for each of the four tested light intensities a higher percentage of 7-day-old bees responded positively to the light source in comparison to 1-day-old bees (see Fig. 3). Prior light treatment does not significantly alter positive phototaxis in 1-day-old bees, but a trend for decreased positive phototaxis was found in light-exposed 7-day-old bees. At the lowest intensity (12.5 %), more than twice as many (2.1 fold) 7-day-old dark-kept bees exhibit positive phototaxis compared to 7-day-old light-treated bees. At an intensity of 25 % the difference in positive phototaxis between the two groups decreased to 1.6 fold, and was finally similar at the two highest intensities. We do not have a conclusive explanation for this phenomenon and can only speculate that somehow prior light treatment either reduces the reception or perception of low light intensities, or reduces the motivation for walking towards low light intensities.

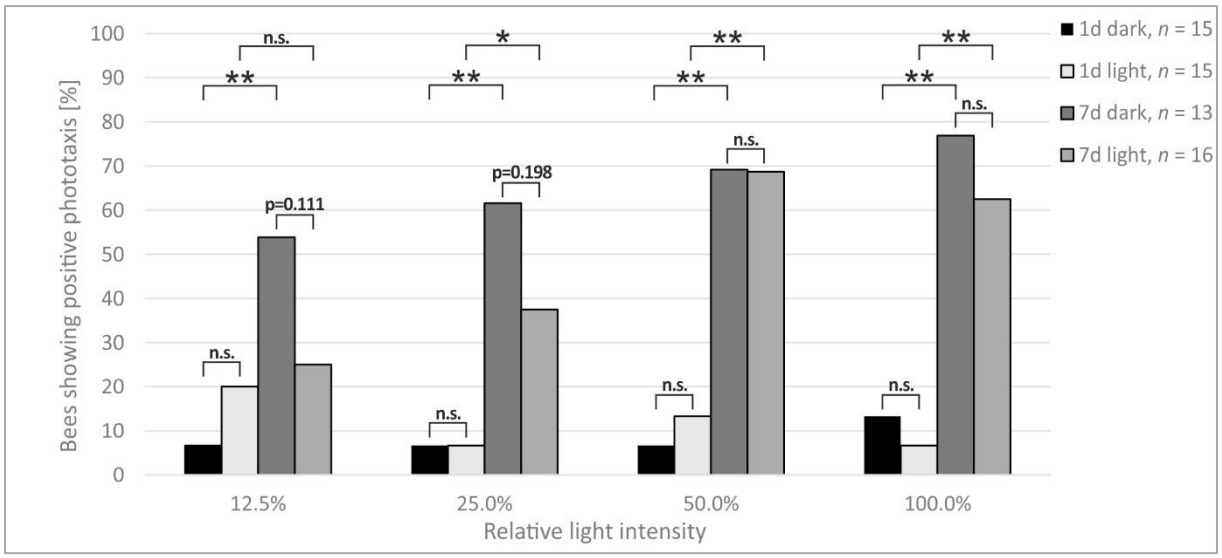


Figure 3: Effect of light and age on phototaxis. 1- and 7-day-old bees which have been exposed to light pulses for 1 day, and age-matched dark-kept control bees were tested for positive phototaxis at four different relative light intensities. 1d, 1-day-old bees; 7d, 7-day-old bees; **P*-value < 0.05; ***P*-values < 0.01; n.s., *P*-value ≥ 0.05.

Discussion

To date very few studies have examined the effects of direct light exposure on gene regulation in the context of neuronal plasticity with the majority of prior work in this field focusing on various aspects of the circadian rhythm. In one relevant study on the light-inducible transcriptome in zebra fish, 117 light-regulated genes have been identified of which most (90) were upregulated (Weger et al., 2011). This is in line with our findings demonstrating an upregulation of 51 genes with only one being downregulated. One possibility for this relatively small number of light-inducible genes is that transcriptional responses to light are chronological with distinct networks activated at different times. This explanation is partly confirmed by the observed age-dependent gene activities. Alternatively, light in general, may affect the expression of a relatively small number of genes. Also, it is likely that different light paradigms and sampling points may result in quite distinct sets of DEGs. For example, the lack of immediate early genes in our dataset, previously reported to respond to light exposure (Tagawa et al., 2005; Nakadate et al., 2013), can be attributed to our specific experimental conditions. Amongst DEGs reported in this paper, *Ip3ka, Cnpy-1, bgm,* or *L(2)efl* participate directly in neuronal plasticity involving neurite outgrowth and synapse morphology (Min, 1999; Bornhauser, 2003; Williams and Mearow, 2011; Windhorst et al., 2012). Thus, these genes could also be critical in mediating dendritic outgrowth in the honey bee brain upon neuronal activation that occurs during the transition from nursing to foraging or after artificial light exposure (Farris et al., 2001; Scholl et al., 2014). This idea is supported by the fact that *L(2)efl* also has been found to be up-regulated in the heads of foragers compared to nurses (Kucharski and Maleszka, 2002a). Interestingly, a study on daily transcript oscillation in *Drosophila* has reported that light-induced transcripts belong to similar broad categories as those identified in our experiments (inositol metabolism, ubiquitin pathway, solute transport) suggesting that in insects light may induce similar molecular responses (Wijnen et al., 2006).

A surprising outcome of our study is the relatively low number of DEGs in the CBr compared to the OLs. The most prominent structural remodeling upon light stimulation occurs in the MBs, manifested by MG pruning (Scholl et al., 2014). We expected this plasticity to be reflected by pronounced transcriptional changes in the CBr, in which the MBs contribute to over 50 % of all cells (Menzel and Giurfa, 2001; Rössler and Groh, 2012). However, as MG elimination in the MBs is due to a pruning of projection neuron boutons which have their cells bodies in the medulla and lobula of the OLs, transcriptional changes reflecting strengthening or weakening of MG may in fact occur in the OLs. Furthermore, the higher number of DEGs in the OLs may indicate severe neuronal plasticity in this region, which so far has not received much attention as it is not as easily quantifiable. However, an

electron microscopy study has revealed synaptic plasticity of photoreceptor neurons in the lamina after manipulation of the visual environment (Hertel, 1983).

Several of our candidate DEGs are part of the epigenetic machinery controlling gene expression either via DNA or chromatin modifications, i.e. histone demethylase *Uty,* histones *H3 and H4* or *Trim71* (Agger et al., 2007; Szenker et al., 2011; Zou et al., 2013). Flexible epigenetic mechanisms modulate coordinated gene expression in a context-dependent manner by acting as the genome-environment interface. For example, histone modifiers like *Uty* have been shown to affect the expression of a number of plasticity-related genes (Agger et al., 2007). Using this mechanism, adult honey bee workers could modulate brain networks to optimize their responses to new environments or to new tasks associated with behavioral maturation, or with light exposure.

We also provide seminal evidence for the role of DNA methylation in regulating light-inducible neuronal plasticity in the honey bee. In insects DNA methylation appears to modulate the transcript levels and also participates in alternative splicing (Foret et al., 2012; Li-Byarlay et al., 2013). Several DEGs identified in this study are known to be methylated, including DEGs with reported plasticity functions like *Cnpy-1*, *Ip3ka, and bgm*. The connection between visual system and DNA methylation dynamics has been confirmed in this study by showing light-induced increases in *bgm* methylation levels. Given the reported role of *bgm* in neuronal plasticity it is likely that the observed methylation changes serve as responsive genomic marks adjusting environmentally-driven expression (Min, 1999; Steinberg et al., 2000). Our findings add to the body of evidence implicating DNA methylation in brain functions in this insect that already includes behavioral transition to foraging (Lockett et al., 2012) and memory formation (Lockett et al., 2010).

Another interesting outcome of our study is a strong age dependence of light-related differences in the transcription of a number of candidate DEGs. For example, the amplitudes of light-induced transcription of *Ip3ka, Cnpy-1, Trim71* and *L(2)efl* are higher in 7-day-old bees compared to 1-day-old bees. One possibility is that these age-dependent differences in transcriptional responses of neuronal plasticity genes to light are important for proper behavioral maturation of adult workers, for example, when they switch to foraging tasks, which is assumed to never happen before they are 4-5 days old (own observations and Schulz et al., 1998). Younger bees may not be developmentally programmed to participate in foraging and their responses to light exposure are predictably less flexible. Indeed, behavioral consequences of age- and environment-dependent gene expression tested by our phototaxis experiments support this notion. A much higher proportion of 7-day-old bees show positive phototaxis compared to 1-day-old bees suggesting that bees at different developmental states exhibit distinct behaviors upon light exposure that correlates with differential expression of relevant neuronal genes. Age-dependent differences in the expression of plasticity-

related genes identified in this study are also apparent when comparing the basal expression levels of the dark-kept control groups between 1- and 7-day-old bees. This result speaks for an endogenous mechanism regulating the chronologic expression of light-responsive neuronal genes during adult maturation. Young bees progress through a series of tasks within the hive which gradually brings them into closer proximity to the hive entrance and light exposure (Winston, 1991). It is likely that this behavior is partly driven by increased phototaxis, and that our observed age-dependent expression of DEGs serves as a molecular regulation of this behavior.

Our findings also complement recent discoveries implicating miRNAs in brain function. The expression of one miRNA, *miR-932,* is affected by light. This miRNA was previously shown to have an effect on long-term memory formation in the honey bee possibly by its direct interaction with the actin gene *Act5c* (Cristino et al., 2014). We have predicted one additional potential target of *miR-932,* namely *Doublecortin* (*GB44947*) that also is known to interact with f-actin (Fu et al., 2013), strengthening the idea that *miR-932* participates in structural plasticity via its interaction with the cytoskeleton at the level of synapses. These small epigenetic regulators are considered proximate factors mediating age-dependent differences in the amplitude of light-induced transcription of neuronal genes. In *C. elegans,* the reciprocal inhibition of *Trim71* and *miR let-7* depends on age and ultimately determines different degrees of axonal plasticity at different ages (Zou et al., 2013). Based on the age-dependent negative correlation between *Trim71 and miR let-7* levels uncovered in our study, a similar mechanism controlling the onset or degree of neuronal plasticity in an age-dependent manner seems possible in the honey bee brain. Indeed, it has been suggested that differentially expressed miRNAs, including *miR let*-7, have a role in developmentally regulated behavioral changes in the honey bee during the transition from nursing to foraging (Behura and Whitfield, 2010). We propose that one role of *miR let-7* and possibly other miRNAs in this behavioral transition involves the refinement of brain networks in expectation of foraging, or after orientation flights when they collide with the external world. This idea is strengthened by the fact that in the honey bee miRNAs are predicted to predominantly target neuronal genes (Ashby et al., 2016).

The specific roles of cellular responses to light are certain to be complex, likely warranting years of future research. The findings presented here signify the importance of investigating dynamic regulation of both gene expression and epigenetic modifiers in behavioral changes brought about by the perception of environmental stimuli. The honey bee system allows an unparalleled experimental transition, from transcriptomes and epigenomes to neural circuitry to sophisticated behaviors, all under entirely natural environmental conditions.

Chapter II

Characterization of *DNMT3* and *TET* expression, and of DNA methylation in the brains of honey bee nurses and foragers

Abstract

In spite of booming interest in methylomics, DNA methylation toolkits in non-mammalian lineages remain mechanistically unexplored hampering both functional and evolutionary explanations of epigenomic control systems in organisms such as insects. Here we investigate the expression and intracellular compartmentalization of the honey bee putative *de novo* DNA methyltransferase AmDNMT3. Furthermore, we examine DNA methylation patterns of the neuronal plasticity related gene *bubblegum* (*bgm*), as well as of a gene associated with circadian rhythm, *timeless2* (*Tim2*). We demonstrate that the DNA methylation patterns of these two genes differ between nurses and foragers, indicating a role of DNA methylation in neuronal and behavioral plasticity in the honey bee. Moreover, we confirm that AmDNMT3 is expressed in the brain, albeit at much lower levels than AmTET that has been implicated in demethylation. Contrary to expectations AmDNMT3 protein is almost entirely cytoplasmic. Our findings suggest that the functionality of this protein in honey bees, including hitherto unidentified substrates, needs to be experimentally reassessed before its role in development and behavioral plasticity can be fully understood.

This chapter is adapted from the unpublished manuscript:

Becker N, Maleszka R, Rössler W. 2016. Honey bee relative of mammalian DNA methyltransferase 3 (DNMT3) is predominantly localised to the cytoplasm in brain neurons. unpublished

Introduction

DNA methylation is an ancient and widely used epigenomic modification that has been implicated in various cellular processes including regulation of gene expression, transposon silencing, chromatin structure and dynamics, embryonic reprogramming, and in creating environmentally-induced epigenomic signatures (Law and Jacobsen, 2010; Feil and Fraga, 2012; Jones, 2012). In mammals, two classes of enzymes are involved in establishing and maintaining genomic methylation patterns. Three paralogs of DNA methyltransferase (DNMT3a, 3b and a regulatory form 3L), play a role in *de novo* methylation, almost exclusively in the CpG context, whereas DNMT1 is deemed to maintain the DNMT3-generated patterns through cell divisions (Jurkowska et al., 2011a). DNMTs 3a and 3b are very similar in structure and function, but differ greatly in their expression patterns that imply that DNMT3b is needed during early development, whereas DNMT3a is required for proper cellular differentiation (Moore et al., 2013). Unlike DNMT1, both DNMT3a and DNMT3b have the capacity to methylate DNA with no preference for hemimethylated DNA (Moore et al., 2013). *DNMT1* and *DNMT3b* genes show embryonic lethal phenotype when mutated, whereas *DNMT3a* knockout mice survive only for approximately four weeks after birth (Okano et al., 1999). The third paralog DNMT3L is catalytically inactive, but stimulates methyltransferase activity by associating with DNMT3a and DNMT3b (Jurkowska et al., 2011a). In recent times this classic separation of *de novo* and maintenance functionalities of DNMT3s and DNMT1 has been replaced with new models following studies suggesting that DNMT1/DNMT3s activities, DNA replication rate, and demethylases cooperatively determine the local methylation status (Jeltsch and Jurkowska, 2014). Recent evidence suggests that DNMT3a and DNMT1 functionally cooperate during *de novo* methylation of DNA and DNMT3s have a role in maintenance. This process also involves chromatin and histone modifiers (Jeltsch and Jurkowska, 2014). Mammalian DNMT3a exists in a self-inhibitory inactive form that requires DNMT3L and acetylation of the N-terminus of histone H3 for catalytic stimulation (Hu et al., 2009; Zhang et al., 2010; Jurkowska et al., 2011a; b; Guo et al., 2014). Furthermore, it has been proposed that histone H2AK119 ubiquitination could also lead to ubiquitin interacting motif UIM-dependent recruitment of DNMT1 and DNA methylation beyond classic maintenance (Qin et al., 2015).

In contrast to mammals, very little is known about the mechanism of DNA methylation in invertebrates. Although proteins with sequence similarity to mammalian DNMTs, evidently capable of converting cytosine to 5-methylcytosine (5mC) are encoded by genomes of most invertebrates, the distribution of the DNA methylation toolkit in this group of animals is quite intriguing (Lyko and Maleszka, 2011). Some lineages, namely *Diptera*, *Placozoa* and many nematodes have lost DNA methylation enzymology and in other lineages the distribution of DNMTs is mosaic with some species

having a combination of one or more paralogs of DNMT1 and one DNMT3, and others missing DNMT3 and having only one DNMT1 (Lyko and Maleszka, 2011; Dabe et al., 2015). It is hypothesized that in organisms missing predicted proteins similar to DNMT3 both *de novo* and maintenance reactions could be carried out by DNMT1-like enzymes, but experimental evidence is still lacking (Lyko and Maleszka, 2011; Falckenhayn et al., 2013). Indeed, the topic of biochemical and cellular properties of these enzymes in insects and other invertebrates remains unexplored.

We have been using the social honey bee *Apis mellifera,* in which the presence of genes encoding proteins with sequence similarities to mammalian DNMTs was reported a few years ago (Wang et al., 2006; Maleszka, 2008), as an insect model for methylomics (Lyko and Maleszka, 2011; Maleszka, 2014). Like in mammals, DNA methylation in the honey bee is symmetrical and occurs almost exclusively at CpG dinucleotides, but the genome is only sparsely methylated within gene bodies. Genome-wide mapping of methyl marks in *Apis* uncovered only ~70,000 5mCs associated predominantly with conserved genes, often spanning splice sites and alternatively transcribed cassette exons (Lyko et al., 2010; Foret et al., 2012). This number stands in stark contrast to over 25 million methylated CpGs in a mammalian genome (Bird, 2002; Smith and Meissner, 2013). The honey bee genome encodes two DNMT1-like and one DNMT3-like proteins that are expressed ubiquitously throughout early development and in adults (Wang et al., 2006; Wojciechowski et al., 2014). A relatively high level of DNMT3 expression in comparison with DNMT1 in post-mitotic brain tissue is consistent with the proposed role of DNA methylation in neuronal plasticity in animals including insects (Wang et al., 2006; Day and Sweatt, 2010; Lockett et al., 2010; Guo et al., 2011). Interestingly, in mammals it is DNMT1 rather than DNMT3*s* that is highly expressed in the central nervous system, although both enzymes have overlapping roles, and changes in synaptic plasticity have been observed only with a double DNMT1/DNMT3A knockout (Inano et al., 2000; Feng et al., 2010). The honey bee also has a single orthologue of the TET/JBP family of 2-oxoglutarate- and iron-dependent dioxygenases that has can hydroxylate 5mC to form 5-hydroxymethylcytosine (5hmC) (Wojciechowski et al., 2014). However, a very high abundance of AmTET mRNAs in the brain cannot be reconciled with a scarcity of 5hmC detected in various tissues in this insect. While these findings generate much interest in insect methylomics, they also highlight an urgent need for more exhaustive analysis of poorly understood epigenetic machineries in non-mammalian organisms.

Here we analyze both the expression and subcellular localization of DNMT3 in the *Apis* brain using qPCR and protein immunohistochemistry, and examine DNA methylation patterns of two neuronal and behavioral relevant genes using bisulfite sequencing with MiSeq (BS-MiSeq). We show that these two genes (*bgm* and *Tim2*) are differentially methylated between nurses and foragers. Furthermore, AmDNMT3 transcripts are expressed in the brain with increased levels found in older workers, but its

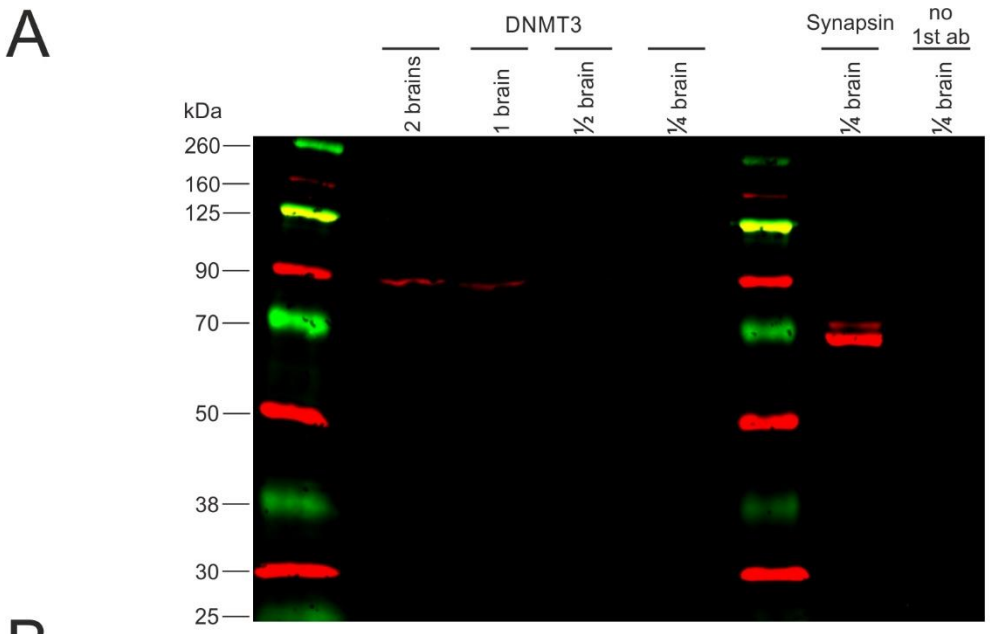
levels of expression are significantly lower than those of AmTET. AmDNMT3 protein localizes to cytoplasm with only sporadic labelling observed in the nucleus. We discuss this unanticipated distribution of AmDNMT3 in the honey brain in the context of the postulated role of this enzyme in *de novo* methylation in post-mitotic neurons.

Materials and methods

Antibody characterization

The mouse anti-DNMT3 antibody used in this study was purchased from Novus biological (64B1446). The epitope is near the C-terminus spanning amino acids 705-908 (Chen, 2002). This region is 44.4 % identical and 62.8 % similar to the honey bee DNMT3 (Fig. 1). The feasibility of using the mouse antibody for the honey bee was evaluated by a semidry Western blot using extracts from brains of foraging bees obtained from the apiary at the Biocenter, University of Würzburg, in June 2015. The bees were snap frozen in liquid nitrogen and their brains dissected in NTE buffer (50 mM NaCl, 25mM Tris, 5 mM EDTA, pH 8) as per our standard protocol (see a detailed video at https://db.tt/wSj9BBxL), homogenized in Laemmli buffer (100 mM Tris/HCl (pH 6.8), 10 % SDS, 10 % β-mercaptoethanol, 30 % Glycerol, 1 % Bromphenol blue) with a 5mm steel bead (Qiagen) on a Tissue Lyser LT (Qiagen) for 5 min at 40 Hz. The homogenate was heated to 95 °C for 5 min and transferred immediately on ice. The samples were subjected to polyacrylamide gel electrophoresis and blotted as previously described with a few modifications (Scholl et al., 2015). Briefly, electrophoresis was run in a 4 % stacking- and 10 % separating gel with a pre run at 80 V for 25 min and at 120 V for 1 h 45 min (running buffer: 250 mM Tris, 1.9 M glycine, 1 % SDS, pH 8.3). Proteins were blotted onto a nitrocellulose membrane (PeqLab: 39-1010) for 2 h at 2 mA/cm^2 (blotting buffer: 25 mM Tris, 192 mM glycine, 20 % methanol, 0.1 % SDS). The membrane was rinsed in TBS and blocked in LI-COR Odyssey® Blocking Buffer TBS overnight at 4 °C. Next, the antibody against DNMT3 was diluted 1:2000 and the antibody against Synapsin (SYNORF1, kindly provided by E. Buchner, University of Würzburg, Germany; for antibody characterization see Groh et al. 2012) 1:4000 in blocking buffer and incubated overnight at 4 °C. For the negative control no primary antibody was added to the blocking buffer. The membrane was washed three times 10 min in TBST (TBS, 0.1 % Tween 20) before the secondary antibody (Goat anti-Mouse IRDye® 680RD, LI-COR: 925-68070) was applied in a 1:20,000 dilution for 1 h at RT with gentle agitation. The membrane was washed three times 10 min with TBST, rinsed with TBS and finally analyzed with the Odyssey Infrared Imaging System (LI-COR). As shown in Figure 1, the mouse anti-DNMT3 antibody detects a clear-cut band of approximately 90 kD in the honey bee brain extracts. This value is in good agreement with the predicted size of AmDNMT3 based on the data in Wang et al. 2006 and the current NCBI gene model NP_001177350 (Fig. 1). There is no putative nuclear localization motif in the translated polypeptide.

No signal is detected with lower protein concentrations or in the negative control. The positive control with Synapsin yields a comparably stronger signal that is expected for an antibody generated against a highly conserved protein from another insect (*Drosophila*).



B

```
M. musculus   705 ------------------------------------------PCNDL 709
                                                            |||||
A. mellifera  501 EIDQDALMVTASHFGDRILQLGNVKDITCNTIKEIAPIDLLIGGSPCNDL 550

              710 SIVNPARKGLYE--GTGRLFFEFYRLLHDARPKEGDDRPFFWLFENVVAM 757
                  |:.||||.|||:  |||.||||:.|:|...| |..::|..|||:|||.:|
              551 SLANPARLGLHDPRGTGVLFFEYRRILKLVR-KLNNERHLFWLYENVASM 599

              758 GVSDKRDISRFLESNPVMIDAKEVSAAHRARYFWGNLPGMNRPLASTVND 807
                  ....:.:|::.|...|.:||:.:.|..||.|.:|.|.|...|.|:|  ..
              600 PSEYRLEINKHLGQEPDVIDSADFSPQHRLRLYWHNFPIEPRLLSS--QR 647

              808 KLELQECL-EH-GRIAKFSKVRTITTRSNSIKQ--GKDQHFPVFMNEKED 853
                  :.::|:.| .| .|.:...|:||:||:.||:||  ||....|:.|.::.|
              648 EQDVQDILTPHCQRYSLVKKIRTVTTKVNSLKQGDGKLALKPILMKDESD 697

              854 ILWCTEMERVFGFPVHYTDVSNMSRLARQRLLGRSWSVPVIRHLFAPLKE 903
                  .||.||:|.:||||.|||||.|:|...||||:|:||||..:..:|..|..
              698 SLWITELEEIFGFPRHYTDVKNLSATKRQRLIGKSWSVQTLTAIFESLCP 747

              904 YFACV------    908
                  :|...
              748 FFERDIVEIEG    758
```

Figure 1: Evaluation of DNMT3 antibody. (A) Western blot analysis of DNMT3 antibody on homogenates from 2, 1, ½ and ¼ honey bee brains, plus a control with a Synapsin antibody and a negative control with no primary antibody. A signal for the DNMT3 antibody is detected just below the 90 kDa mark, which is at the expected size of AmDNMT3 based on the current gene model (GenBank NP_001177350). The intensity of the signal is concentration dependent. (B) Pairwise protein alignment of the mouse DNMT3 sequence spanning the antibody epitope and the honey bee DNMT3 sequence reveals 44.4 % identity and 62.8 % similarity. 1st ab, primary antibody.

Fluorescence immunohistochemistry

Nurses and foragers from the apiary of the Biocenter, University of Würzburg were collected in July 2015, and in February and April 2016, and their brains were dissected for fluorescence

immunostaining according to our standard protocol (Muenz et al., 2015). Briefly, fixed brains were embedded in agarose and sectioned at 100 µm in frontal planes. The primary antibody against DNMT3 was diluted 1:100 and incubated for 2 days with gentle agitation at 4 °C. The secondary antibody (CF633 goat anti-mouse lgG(H+L), Biotium: 20121) was applied at a dilution of 1:250 overnight at 4 °C. DNA was marked with Hoechst 34580 (Invitrogen) diluted 1:10,000 in PBS. For Figure 6B, showing an exemplary staining of a forager brain, a stack of 11 images was acquired with a Zeiss Elyra S.1 SIM Microscope and merged to one single image (one image every 100 nm, covering a distance of 1 µm in the Z-axis). In a second protocol, brain slices were treated with 4 N HCl for 10 min at RT prior to antibody incubation to denature DNA to retrieve the DNMT3 antigen from potential masking by its interaction with DNA (Sasaki et al., 1988). In this protocol DNA was labelled with Sytox Green (Molecular Probes) diluted 1:10,000 in PBS and images were acquired with a confocal microscope (Leica TCS SP2, Leica Microsystems).

Single cohort colonies

Single cohort colonies (SCCs) were prepared as described previously (Lockett et al., 2012). Two independent replicates of this experiment were performed with *Apis mellifera ligustica* in Canberra, Australia in February 2015, and with *Apis mellifera carnica* in June 2015 in Würzburg. Eight-day-old young nurses (YN) and young foragers (YF), as well as 22-day-old old nurses (ON) and old foragers (OF) were sampled for qPCR and BS-MiSeq experiments. The behavioral status of nurses and foragers was confirmed during brain dissection by the morphology of their hypopharyngeal glands (Maleszka et al., 2009).

Quantitative real-time PCR

Quantitative real-time PCR and data analysis was performed as described in Chapter I, with the following adjustments. The optic lobes (OLs) and the mushroom bodies (MBs) from three brains of bees from the SCC were pooled per sample and RNA was extracted as per a standard Trizol protocol. Six to eight samples were gained from the Canberra SCC, and four samples derived from the Würzburg replicate, resulting in an overall sample size of 10-12 (indicated in Fig. 2). Forward and reverse primer sequences for AmDNMT3 were TACAAACTGTCGGAGGTGCA and AGCGTCGTCCAAAGTCCAGT, GTCAGTGAGATCAGAGGAGC and TGGTGCAAGGCTGAGGTACA for amTET, as well as CGTCATATGTTGCCAACTGGT and TTGAGCACGTTCAACAATGG for the reference gene *Rpl32*. Relative expression ratios (*R*) were calculated by combining ct-values from samples from both replicates of the SCC.

Bisulfite sequencing with MiSeq

Bisulfite sequencing with MiSeq was performed as described in Chapter I, with the following adjustments. Samples derived only from the Canberra SCC. For each group (YN, YF, ON, OF), 12 MBs

and 12 pairs of OLs, respectively, were pooled. DNA was extracted as per a standard Trizol protocol. The sequence for the outer forward and reverse primers for *bgm* were TTTTTTAATAATTTTAGGTAGTTG/AATAAATACTTACTTCAAATTTAC, and GCAGAATTCTATTTTATGTTATATATAGTTGGT/CGCAAGCTT-CTAATATATTCACAATATATACAC for the forward and reverse nested primers. For *Tim2* outer primer sequences were TTATTAAATATTTAAGAAGAGATG/TACAAACAAAACATATTCAATATC and GCAGAATTCTTTGGGATGTATTATTAAGGTAAG/CGCAAGCTTTACCTCATCATAAATTATAACATC for the forward and reverse nested primers.

Results and discussion

Task-related expression of AmDNMT3 and AmTET in the brain

To disentangle the effect of age, experience and task on the DNA methylation machinery in different areas of the honey bee brain we have quantified the expression of AmDNMT3 and AmTET transcripts in individuals from single cohort colonies in which age-matched bees perform either nursing or foraging duties (Robinson et al., 1989). In the mushroom bodies (MBs), a brain region associated with cognitive functions (Giurfa, 2007; Hourcade et al., 2010), a slight trend for a lower expression of both AmDNMT3 and AmTET is noticeable in young foragers (YF) compared to young nurses (YN) (Fig. 2). In the MBs of older bees the expression of AmDNMT3 is significantly higher in foragers than in nurses and also a trend for a higher expression of AmTET is apparent. No clear differences are seen between young and old nurses. In the optic lobes (OLs), gene expression levels between the four groups differ only for old foragers, where AmTET and AmDNMT3 are significantly upregulated. No differences between young and old nurses are observed in both compartments, but tasks rather than chronological age influence the expression of these genes in mature honey bee workers. The differential expression of AmDNMT3 and AmTET in the MBs between young and old foragers suggest an experience-related response. Older foragers are likely to have experienced a longer foraging history associated with various environmental stimuli, cognitive challenges and intense physical and metabolic activity. Hence, the upregulation of AmDNMT3 and AmTET may represent an adaptation of the methylation machinery to an extended foraging activity. This idea is supported by previous findings reporting changes in DNA methylation in response to external stimuli and foraging experience (Day and Sweatt, 2010; Guo et al., 2011; Herb et al., 2012; Lockett et al., 2012).

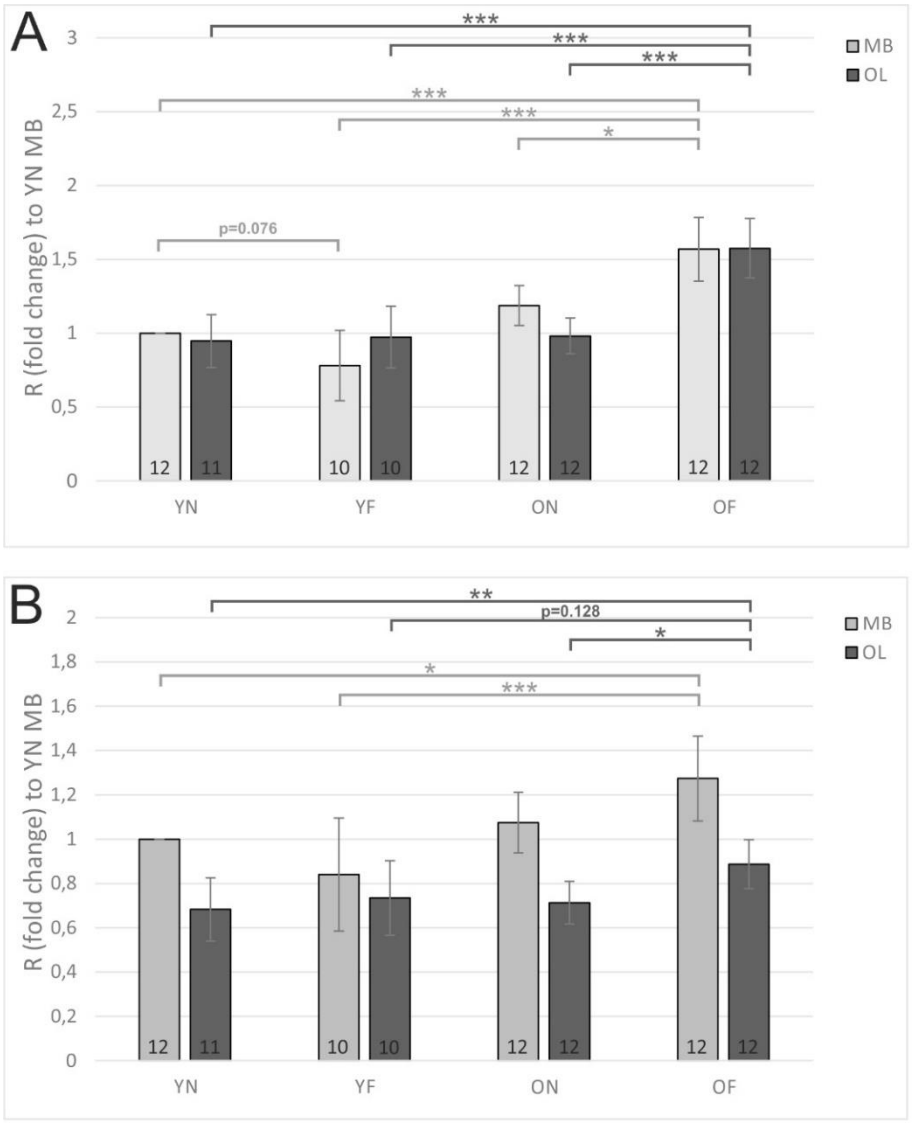


Figure 2: Relative gene expression ratios of *DNMT3* and *TET* between honey bees of different ages and worker castes. Gene expression levels of *DNMT3* (A) and *TET* (B) in the MBs and OLs are highest in 22-day-old OF compared to age-matched ON, and to 8-day-old YN and YF. For the MBs the lowest expression of *DNMT3* and *TET* is found in YF, whereas no clear difference is observed between YN and ON. In the OLs expression levels are similar for YN, YF, and ON. The experiment was performed two times independently from each other, once in Canberra and once in Würzburg. Six to eight samples were gained from the Canberra replicate, and four samples derived from the Würzburg replicate. For the ratio calculations the ct-values from samples from both experiments were combined. *R*, relative expression ratio; MB, mushroom body; OL, optic lobe; YN, young nurses (8-day-old); YF, young foragers (8-day-old); ON, old nurses (22-day-old); OF, old foragers (22-day-old); **P*-value < 0.05; ***P*-values < 0.01; *** *P*-values < 0.001; n.s., *P*-value ≥ 0.05.

AmTET expression in the brain is much higher than AmDNMT3 expression

AmTET transcripts are five to six times more abundant in the MBs and three to four times more abundant in the OLs than AmDNMT3 mRNAs (Fig. 3). Given the scarcity of 5hmC in this species, this very high level of AmTET brain expression supports the notion that besides converting 5mC to 5hmC, AmTET has to perform other functions in the honey bee brain (Wojciechowski et al., 2014). In addition to various regulatory roles of mammalian TETs including recruitment of histone modifying complexes (Xu et al., 2012; Pastor et al., 2013), recent data also implicate this enzyme in 6-adenine

methylation and mRNA hydroxymethylation in *Drosophila* (Zhang et al., 2015; Delatte et al., 2016; Maleszka, 2016). Whether these regulatory and extra catalytic activities are also typical of honey bees and other insects' TETs needs to be established experimentally.

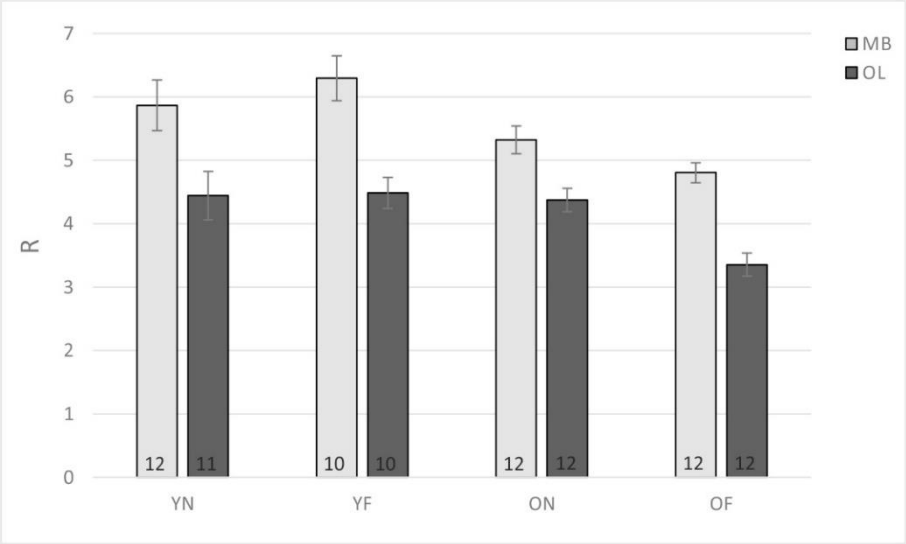


Figure 3: Relative expression of *TET* compared to *DNMT3* in the brains of honey bees of different ages and worker castes. *TET* is three to six times higher expressed than *DNMT3*. *R*, relative expression ratio of *TET* to *DNMT3*; MB, mushroom body; OL, optic lobe; YN, young nurses (8-day-old); YF, young foragers (8-day-old); ON, old nurses (22-day-old); OF, old foragers (22-day-old).

Two neuronal- and behavioral relevant genes are differentially methylated between nurses and foragers

Methylated genes of the honey bee are predominantly associated with physio-metabolic processes (Foret et al., 2012; Ashby et al., 2016). Thus, it seems possible that the high expression of *DNMT3* and *TET* in experienced foragers provides an epigenetic mechanism adapting the metabolism to foraging activity. Accordingly, we report differences between nurses and foragers in the DNA methylation pattern of *bgm* (Fig. 4), a gene involved in brain long-chain fatty acids metabolism, myelinogenisis, and the correct formation of the optic lobes in adult flies (Min, 1999; Steinberg et al., 2000). This gene is also reported to be differentially methylated between light-exposed and dark-kept honey bees in Chapter I, and therefore qualifies as a good illustrator for neuronal adaptations to the environment. Furthermore, *Tim2* is differentially methylated between nurses and foragers (Fig. 5). This gene is thought to adjust the circadian rhythm to the actual day length in *Drosophila* (Benna et al., 2010). Whereas nurses are active around the clock, foragers exhibit a circadian rhythm where they are active during the day and rest at night (Bloch, 2010). Circadian behavior was shown to be reprogrammed by plastic DNA methylation in mice (Azzi et al., 2014). Possibly a similar mechanisms exist in the honey bee as indicated by the differential methylation of *Tim2* between nurses and foragers. Perhaps the plastic methylation of this gene is import for the control of circadian behavior in the honey bee.

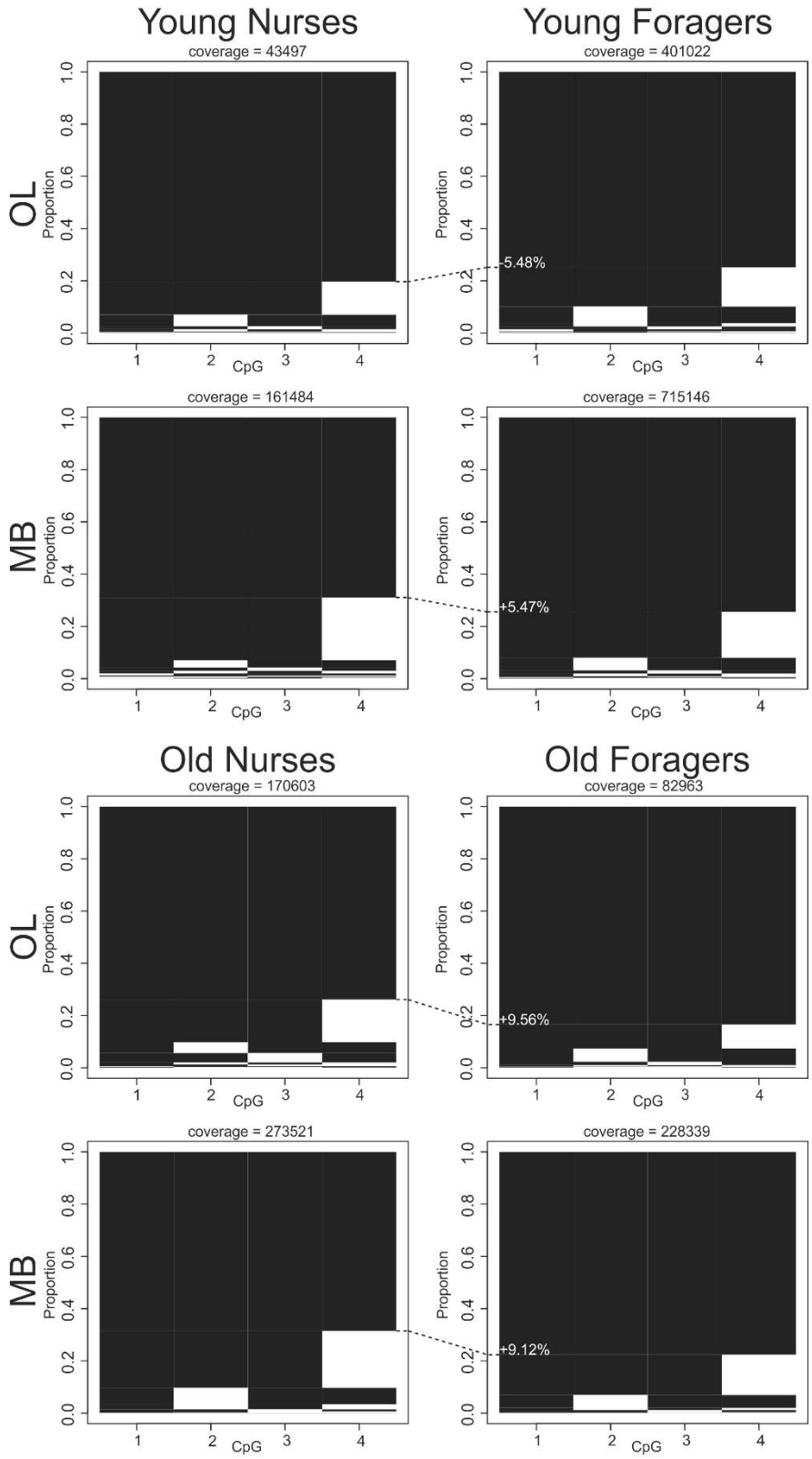


Figure 4: Brain methylation patterns of *bgm* in honey bees of different ages and worker castes. Methylation patterns in *bgm* revealed by deep amplicon sequencing. Each row represents a methylation pattern (black: methylated CpGs, white: not methylated CpGs), the height of each pattern is proportional to the pattern's abundance. *bgm* amplicons were amplified from both OLs and MBs of young nurses and foragers (8-day-old), and of old nurses and foragers (22-day old) from a single cohort colony. After normalizing pattern frequencies several distinct and highly abundant methylation patterns have been detected. The pattern proportions are sorted from the most abundant at the top to the least abundant at the bottom. The number of sequenced reads for each situation is shown above each panel. OL, optic lobes; MB, mushroom bodies.

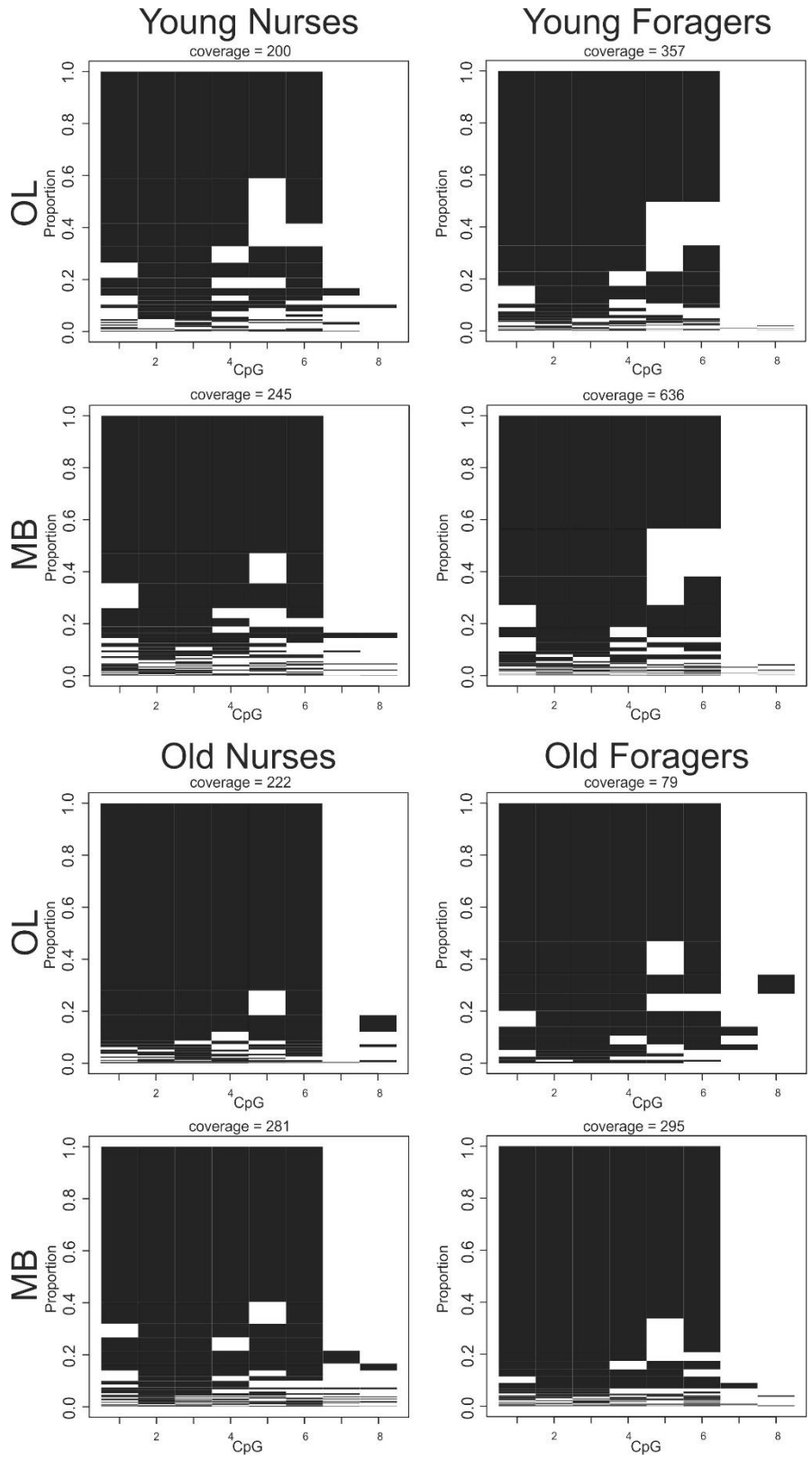


Figure 5: Brain methylation patterns of *Tim2* in honey bees of different ages and worker castes. Methylation patterns in *Tim2* revealed by deep amplicon sequencing. Each row represents a methylation pattern (black: methylated CpGs, white: not methylated CpGs), the height of each pattern is proportional to the pattern's abundance. *Tim2* amplicons were amplified from both OLs and MBs of young nurses and foragers (8-day-old), and of old nurses and foragers (22-day-old) from a single cohort colony. After normalizing pattern frequencies several distinct and highly abundant methylation patterns have been detected. The pattern proportions are sorted from the most abundant at the top to the least abundant at the bottom. The number of sequenced reads for each situation is shown above each panel. OL, optic lobes; MB, mushroom bodies.

AmDNMT3 protein is predominantly localized to the cytoplasm in brain cells

To determine if there are regional differences in the expression of AmDNMT3 in the brain we used a commercially available DNMT3 antibody to visualize AmDNMT protein compartmentalization. As shown in Figure 6, AmDNMT3 is ubiquitously expressed throughout all brain areas with no apparent regional differences. The staining is generally weak, most likely due to a relatively low abundance of this protein, which is in line with the faint band observed in our western blot (Fig. 1), and the relatively low levels of AmDNMT3 mRNA (Fig. 3). Most interestingly, AmDNMT3 is predominately compartmentalized in the cytoplasm and only rarely found in the nucleus. This is true for all brain regions in both nurses and foragers of undefined ages as illustrated by one example of a forager brain in Figure 6. Given the proposed role of this enzyme in *de novo* methylation, this pattern is surprising. To exclude that this unexpected outcome has technical reasons due to masking of the DNMT3 antigen by its interaction with DNA, an acidic based antigen retrieval was performed to denature DNA. As DNMT3 compartmentalization does not differ between the standard protocol and the protocol with an extra retrieval step (Fig. 7), biological rather than technical reasons have to be considered for the high cytoplasmic abundance of DNMT3. A number of studies have reported cytoplasmic as well as nuclear localization of mammalian DNMT3a, 3b and 3L in cell cultures and gametocytes with no clear pattern emerging (Lees-Murdock et al., 2005; Van Emburgh and Robertson, 2011; Zhang et al., 2011; Agarwal et al., 2013; Guimarães et al., 2015). Only a few studies investigated the subcellular localization of DNMT3s in brain tissue. Feng et al. (2005) detected an exclusive nuclear expression of DNMT3a in various neuronal cells including adult post-mitotic brain cells. In the olfactory epithelium of mice, DNMT3b is found in proliferating progenitors and DNMT3a is expressed in post-mitotic neurons. Both enzymes seem to predominantly localize to the nucleus (MacDonald et al., 2005).

A possible relevance of the cytoplasmic abundance of DNMTs is poorly understood. In aging mice oocytes a cytoplasmic-nuclear trafficking of the enzyme was described (Zhang et al., 2011). It has been suggested that such a mechanism provides more precision in regulating DNA methylation during specific time windows important for normal development (Smith and Meissner, 2013). The high abundance of AmDNMT3 in the cytoplasm of adult brain neurons in honey bees may play a regulatory role outside the developmental context. For example, shuttling a catalytically inactive enzyme from the cytoplasmic storage could ensure rapid responses to stimuli that require DNA methylation without burdening the transcriptional and translational machineries. A most likely possibility is that in non-dividing neurons only a sporadic *de novo* methylation is required, for example during memory processing. Such intermittent activity would be associated with a small number of neurons that process and store a particular memory trace. A few nuclei labelled in the MBs may in fact represent these activity-driven events (Fig. 6).

A high flexibility of *de novo* methylation could also be important for behavioral plasticity such as switching from nursing to foraging and vice versa. A link to changes in DNA methylation during this process has been proposed (Herb et al., 2012; Lockett et al., 2012). Interestingly, this transition is associated with structural remodeling in the brain (Withers et al., 1993; Farris et al., 2001; Groh et al., 2012; Muenz et al., 2015) in which DNA methylation could be involved in a similar manner to that found in mice were synaptic plasticity relies on the presence of DNMTs (Feng et al., 2010).

Finally, the predominant cytoplasmic presence of DNMT3 might be related to other catalytic activities involving non-DNA substrates. 5mC has also been found in both prokaryotic and eukaryotic mRNAs and non-protein coding RNAs (Motorin et al., 2010; Cantara et al., 2011; Squires et al., 2012). Although RNA modifications are catalyzed by specialized enzymes (Squires et al., 2012; Popis et al., 2016), the recent discovery in *Drosophila* that TET can hydroxymethylate mRNAs (Delatte et al., 2016) suggests that enzymes thought to be involved in DNA methylation/demethylation may have additional properties not necessarily related to DNA. Modern DNMTs have evolved from RNA-modifying enzymes (Iyer et al., 2011) and some of their ancestral properties still may be used in certain organisms. Cleary, this idea is worthy of further investigation as part of efforts to fully understand the role of all three putative DNMTs in behavioral plasticity and development.

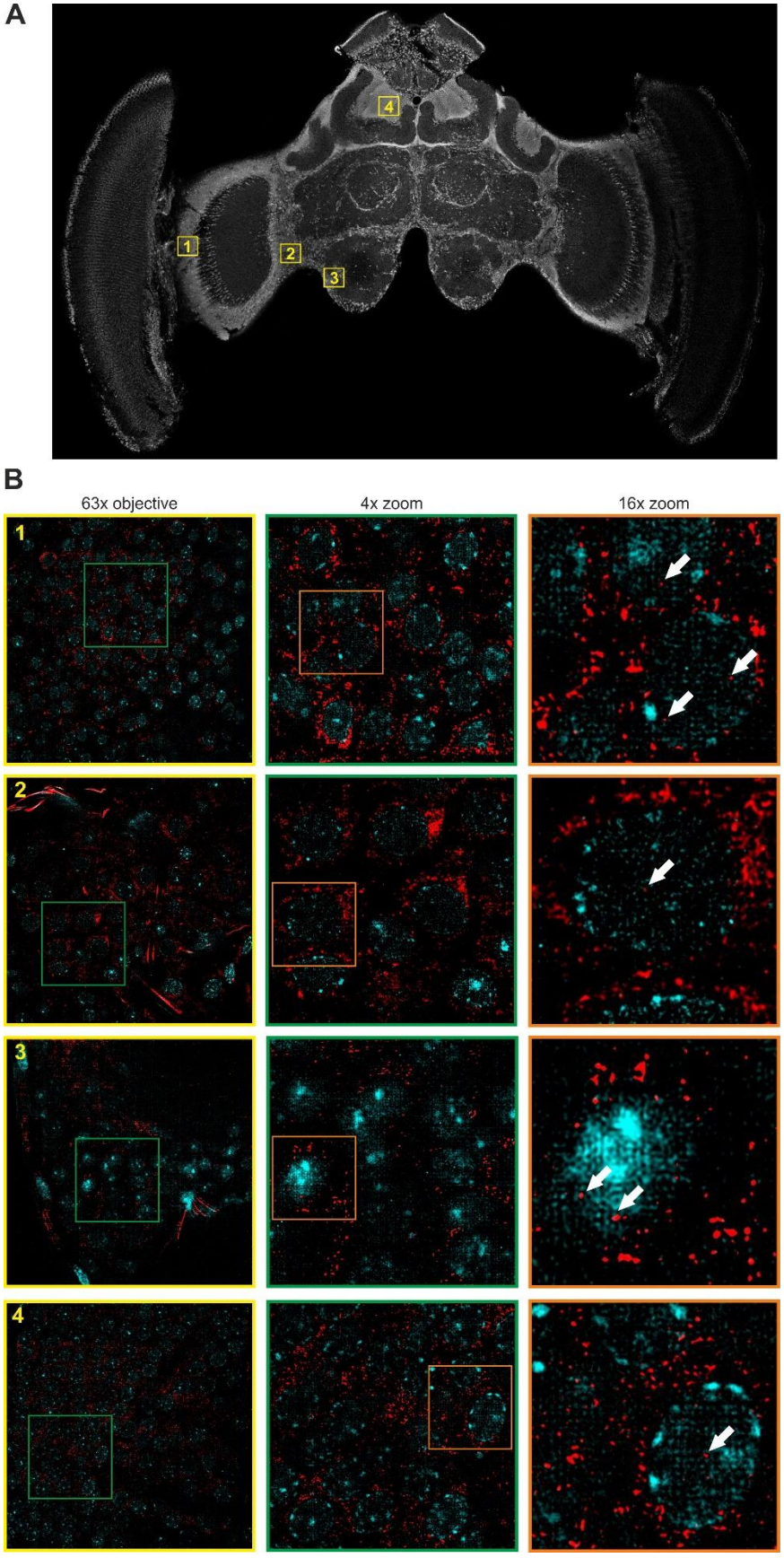


Figure 6: Subcellular localization of AmDNMT3 protein in the honey bee brain. (A) Hoechst nuclear staining of a forager brain section used for the higher magnification analysis in B. (B) Staining against AmDNMT3 in red and against DNA with Hoechst in blue in the optic lobe (1 and 2), in the antennal lobe (3), and in the mushroom body calyx (4). AmDNMT3 signal is almost exclusively detectable in the cytoplasm with only very rare labelling found in the nucleus (indicated by white arrows).

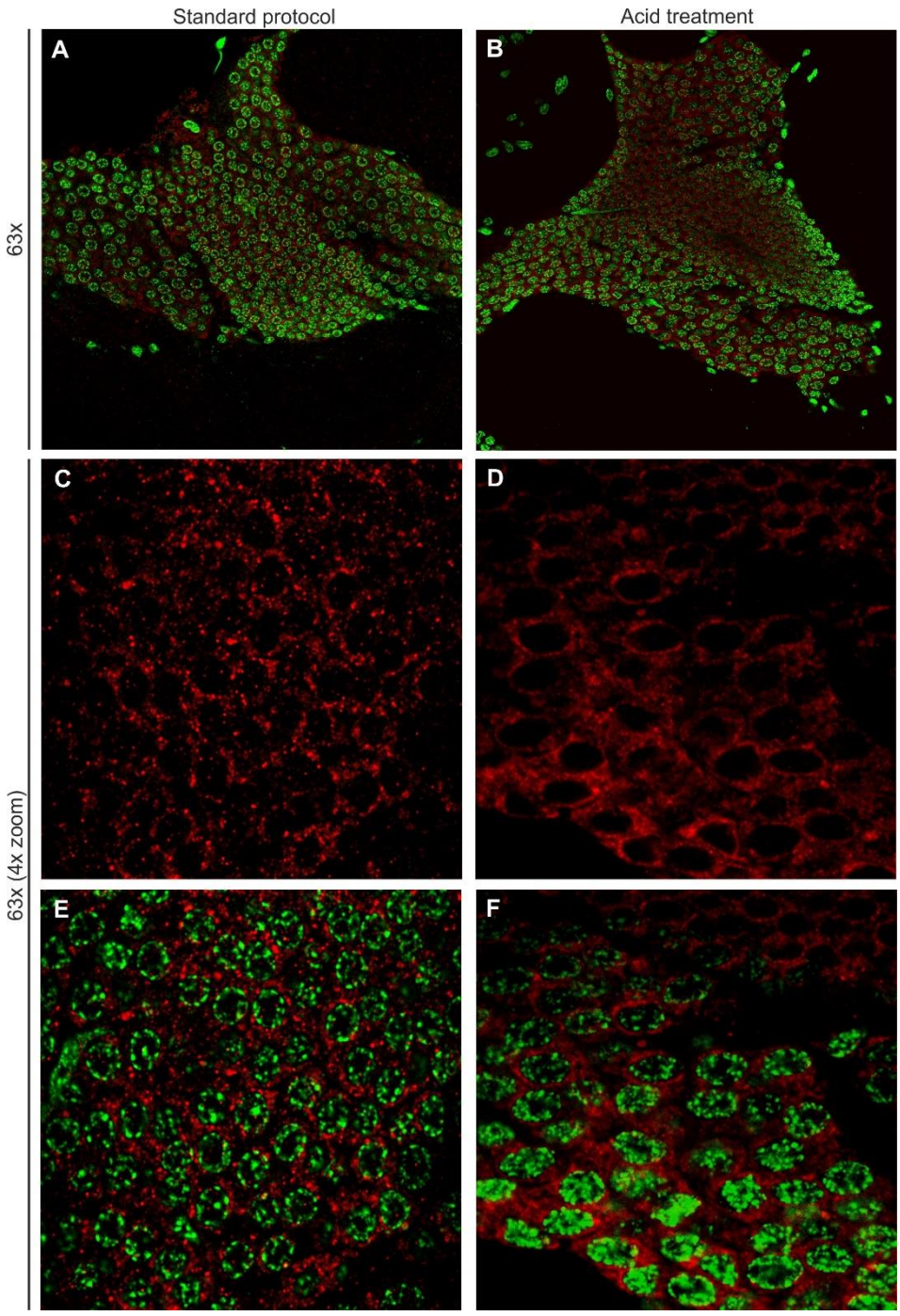


Figure 7: Comparison of immunohistological DNMT3 staining protocols with and without acid antigen retrieval. A, C and E show a staining with the standard protocol, whereas images B, D, and F derived from a protocol with an additional acid antigen retrieval step. A and B give an overview of the mushroom body calyx. As shown in C and D, higher magnification of Kenyon cells under both protocols exhibits a strong cytoplasmic signal of DNMT3, which is nearly absent in the nucleus. E and F provide a merge of DNMT3- and DNA signal. Red: DNMT3 staining. Green: Sytox Green DNA staining.

General Discussion

The phenomenon of environmentally-induced neuronal plasticity has been investigated for several decades, but still many questions concerning the molecular underpinning remain unclear. One urging question is how different molecular pathways like gene transcription and epigenetic gene regulation by miRNAs and DNA methylation work together to remodel the brain. The present thesis aims at bringing light into this topic by studying molecular changes related to visual-exposure induced plasticity in the honey bee brain.

Plasticity-related genes respond to light exposure in the optic lobes and central brain

Visually-induced plasticity has been most extensively studied in ocular dominance columns of mammals (reviewed in Kiorpes, 2015). Examples of structural remodeling of the brain upon simple light exposure have also been found in Amphibia and insects (Sin et al., 2002; Stieb et al., 2010; Scholl et al., 2014). A few prominent plasticity-related genes and proteins like *c-Fos*, *Arc*, cAMP, PKA, Creb and Rho GTPases are thought to be involved (Mower et al., 2002; Sin et al., 2002; Tagawa et al., 2005; Yuan et al., 2011; Nakadate et al., 2013; Frantz et al., 2016). To date, only a few studies have analyzed the whole transcriptome in response to light exposure and the majority of these publications are in the context of various aspects of circadian rhythm (for example Wijnen et al., 2006; Weger et al., 2011; Ben-Moshe et al., 2014; Adewoye et al., 2015). Nonetheless, comparing the results of this thesis with two relevant studies from that area reveals important similarities. In zebrafish, only 117 light-induced genes have been identified, of which the majority (90) was upregulated (Weger et al., 2011). This relative small number of differentially expressed genes (DEGs), compared to other transcriptomic studies (for example Zayed and Robinson, 2012), is in line with results of this thesis, reporting only eight DEGs in the central brain (CBr) and 52 DEGs in the optic lobes (OLs) of which all but one gene are upregulated. This suggests that first light exposure may generally affect only a small number of genes. Another possibility is that light exposure induced transcription is chronologic with a different number and set of DEGs being up- or down-regulated at different time points during or after visual stimulation. Therefore, the list of DEGs identified here may be specific to our light program and sampling point. For example, the lack of immediate early genes in this study, as previously reported to be induced by visual stimulation (Tagawa et al., 2005; Nakadate et al., 2013), may be attributed to the specific light exposure paradigm. A second study in *Drosophila* also describes only a relatively small number of light-induced genes, which belong to similar categories like DEGs reported here: inositol metabolism, ubiquitin pathway, and solute transport (Wijnen et al., 2006). Thus, light exposure seems to evoke similar molecular responses in different insects.

Among the 60 light-activated DEGs revealed by RNAseq, some genes have reported direct functions in structural plasticity. For example, the DEGs *Ip3ka, Cnpy-1, bgm,* or *L(2)efl* are involved in synapse morphology and neurite outgrowth (Min, 1999; Bornhauser, 2003; Williams and Mearow, 2011; Windhorst et al., 2012) and may thus participate in light-induced structural remodeling of the honey bee brain. Furthermore, they could also contribute to the dendritic outgrowth observed in the mushroom bodies (MBs) during the transition from nursing to foraging (Farris et al., 2001). The detection of plasticity genes shows the successfulness of this experimental approach in identifying candidate genes for light-induced structural plasticity in the honey bee brain. This result further bears the future perspective that any of the 60 identified DEGs so far not reported to function in neuronal plasticity may have a role in this context.

Ten from the 60 DEGs discovered with RNAseq were analyzed by qPCR with material derived from independent replicates of the experiment. Seven genes show the same tendency of change in both experimental sets, resulting in 70 % reproducibility. Interestingly, a transcriptomic study investigating changes during the transition from nursing to foraging also reported a 70 % reproducibility rate (Lutz et al., 2012), suggesting a general variability in gene expression in the honey bee. The 30 % discrepancy in the present thesis, however, may be well explained by differences in the experimental design. RNAseq studies were carried out in Canberra, Australia with *Apis mellifera ligustica*, whereas qPCR studies were performed in Würzburg, Germany with *Apis mellifera carnica.* Given that extensive brain gene expressional differences between *ligustica* and *carnica* races were reported (Whitfield et al., 2006), the high reproducibly rate of 70 % indicates a strong conservation of transcriptomic profiles in response to light exposure between both races.

Another surprising outcome of this thesis is the relatively low number of only eight DEGs in the CBr compared to 52 DEGs in the OLs. One most prominent structural change upon light exposure is manifested in MG pruning in the MBs (Scholl et al., 2014). This plasticity was expected to be reflected by higher transcriptomic changes in that brain region. Possibly, at other sampling points after light stimulation a more severe transcriptional effect would be visible. Alternatively, as MG pruning in the MBs is due to an elimination of synaptic boutons from projection neurons originating in the OLs, with their cell bodies in the medulla and lobula (Groh et al., 2012), transcriptomic changes may rather be present in these brain compartments. The higher number of DEGs in the OLs could also reflect the remodeling of this region in response to visual stimulation. For example, an electron microscopy study has revealed a reduced number of photoreceptor synapses in the lamina upon manipulation of the visual environment of the honey bee (Hertel, 1983). Furthermore, light-induced volumetric increases of the OLs were described for *Drosophila,* the house fly, and the ant *Camponotus rufipes* (Barth et al., 1997; Rybak and Meinertzhagen, 1997; Yilmaz et al., 2016). Similar, yet unrecognized,

volumetric and subcellular changes could also occur in the honey bee, explaining the high number of DEGs in the OLs.

Age influences transcriptional properties of candidate plasticity genes

The expression of a number of plasticity-related DEGs investigated in this thesis is influenced by age. Age affects the amplitude of light-induced gene expression, the basal expression of DEGs in naive animals, and the standard deviation of gene expression between individuals.

Under certain conditions, honey bees are capable of precocious foraging with four to five days of age, but never earlier (own observations, Schulz et al., 1998). Furthermore, in the present phototaxis experiments a much higher percentage of 7-day-old bees is positive phototactic compared to 1-day-old bees. These observations demonstrate the strong influence of age on the behavioral capabilities of bees. Possibly, a developmental delay prevents very young bees from foraging, or even from responding to certain foraging-related stimuli like light. This notion is supported by a delayed experience-independent maturation of the MBs during the first week of adult life (Muenz et al., 2015). Therefore, the molecular response of 1-day-old bees to environmental stimuli associated with outside tasks, like light exposure, may not be as flexible as in older bees. This could explain the higher amplitudes of light-induced expression of the plasticity genes *Ip3ka, Cnpy-1, Trim71* and *L(2)efl* in 7-day-old bees compared to 1-day-old bees.

Comparing the basal expression of DEGs between 1- and 7-day-old dark-kept control bees reveals age-dependent differences. This differential expression of neuronal genes might represent a molecular correlate of age-dependent behavior.

Finally, the standard deviation of expression levels was higher in 7-day-old bees for seven of ten DEGs compared to 1-day-old bees. A similar increase in inter-individual variation was previously described for juvenile hormone titers in honey bees (Scholl et al., 2014). These observations may indicate that the time point around seven days of age is associated with important molecular and behavioral changes resulting in a higher heterogeneity between individual bees. This could have important implications for the regulation of division of labor.

Environmental stimuli induce epigenetic changes possibly involved in neuronal plasticity

A central outcome of this thesis is the identification of epigenetic changes correlating with environmentally-induced plasticity. Among the light-induced DEGs, several have direct epigenetic functions like the histone-associated genes histone demethylase *Uty* (Agger et al., 2007), and histone genes *H3* and *H4* (Szenker et al., 2011). Histone modifications such as acetylation and methylation control DNA accessibility and consequently regulate gene expression (Agger et al., 2007; West and

Greenberg, 2011). Neuronal activity has been shown to lead to histone modifications mediating the expression of plasticity-related genes (West and Greenberg, 2011). The regulation of neuronal plasticity in the honey bee may similarly involve histone modifications, as indicated by the identification of the histone demethylating DEG *Uty*.

Besides histone modifications, DNA methylation has been shown to play a role in neuronal plasticity. In mammals, synaptic plasticity requires the presence of DNMTs (Feng et al., 2010), and a few plasticity-related genes have altered methylation patterns after neuronal activity and after LTP (Guo et al., 2011; Sui et al., 2012). There is evidence that DNA methylation is also important for neuronal plasticity in honey bees, as the inhibition of DNMT3 leads to impairments in learning and memory (Lockett et al., 2010). The present thesis provides further indication for a role of DNA methylation in neuronal plasticity in the honey bee, as some plasticity-related DEGs like *bgm*, *Ip3ka*, or *Cnpy-1* are known to be methylated. Changes of their methylation patterns may represent a mechanism controlling plasticity. Indeed, the methylation pattern of *bgm* is modified in the light exposure paradigm. Furthermore, alterations of DNA methylation may also be in involved in naturally occurring brain plasticity, since in this thesis *bgm* is shown to be differentially methylated between nurses and foragers. The comparison of age-matched nurses and foragers reveals that in addition to age, experience and therefore environmental stimuli determine the methylation pattern of this gene in a natural context. The observed DNA modifications are accompanied by changes of *DNMT3* and *TET* expression levels. Levels are highest in old foragers compared to age-matched nurses, as well as to younger nurses and younger foragers. Thus, the expression of DNA modifying genes seems rather to be regulated by experience than by age in adult bees. Results from this thesis are in line with other studies reporting methylomic differences of the plasticity-related genes *Nadrin* and *iswi* between nurses and foragers (Herb et al., 2012; Lockett et al., 2012). Together, these observations strongly support the idea of an implication of DNA methylation in neuronal plasticity in the honey bee.

miRNAs may also be involved as another gene regulatory mechanism in neuronal plasticity in the honey bee brain. miR-932 is induced in the OLs after light exposure, as shown in this thesis, and has previously been identified to be upregulated in visual and olfactory learning tasks in honey bees (Cristino et al., 2014; Qin et al., 2014). This miRNA may exert its function in plasticity by cytoskeleton remodeling, since it directly interacts with transcripts of the actin coding gene *Act5C* (Cristino et al., 2014). The present thesis further supports a role of this miRNA in cytoskeleton organization, as bioinformatical analysis predict *Doublecortin* as a target of miR-932. *Doublecortin* plays a role in microtubule and f-actin formation (Fu et al., 2013). Another noteworthy predicted target of miR-932 is *Hyperplastic discs*, a gene which is crucial for correct photoreceptor differentiation in *Drosophila* (Lee, 2002). *Hyperplastic discs* could thus be involved in adaptation processes following first light

exposure in the honey bee OLs. However, neither of the three mentioned potential target genes are found to be differentially regulated after light exposure in the present RNAseq experiments. miRNAs are known to subtly fine tune gene expression at distinct subneuronal locations like dendrites (Aksoy-Aksel et al., 2014; Smalheiser, 2014), and our approach of isolating mRNAs of entire brain regions may be too crude to identify minor changes at distinct locations or individual neuropils.

The miRNA miR let-7, although not regulated by light in this study, could also have a function in structural plasticity through its interaction with *Trim71,* one of the DEGs that responded most heavily to light exposure. In *C. elegans*, the reciprocal inhibition of miR let-7 and *lin-41* (the homolog of *Trim71*) determines the degree of axon plasticity in an age-dependent manner (Zou et al., 2013). In young larvae, a high degree of plasticity correlates with high transcription levels of *lin-41*, suppressing the expression of miR let-7*.* With growing age miR let-7 levels increase, leading to a decline of *lin-41* levels and ultimately to a decreased axonal plasticity potential (Zou et al., 2013). In this thesis, a similar age-dependent relationship of miR let-7 and *Trim71* levels is described. In 1-day-old bees, miR let-7 levels are twice as high as in 7-day-old bees, which correlates with nearly half the amplitude of light-induced *Trim71* expression in 1-day-old bees compared to 7-day-old bees. Hence, miR let-7 and *Trim71* may also determine the degree and potential of neuronal plasticity in the honey bee brain in an age-dependent manner. miR let-7 levels could represent a mechanism that chronologically regulates the molecular, neuro-plastic, and behavioral responsiveness of honey bees to environmental stimuli.

New roles of the DNA methylation machinery

As stated above, the present thesis provides seminal evidence for the implication of DNA methylation in environmentally-induced neuronal plasticity in the honey bee brain. However, the precise mechanism by which DNA methylation contributes to neuronal plasticity remains unclear. One reason for this is that the process of DNA methylation itself is not fully understood. The incompleteness of the current model of DNA methylation is illustrated by recently discovered new roles of the mammalian DNA methylation enzymes (Jeltsch and Jurkowska, 2014). Traditionally, DNMT3s were thought to mediate the canalization of new methylation marks on CpGs, whereas DNMT1 was supposed to be deemed to the maintenance of these epigenetic marks. Nowadays, it is appreciated that this strict categorization does not count anymore, but that DNMT3s and DNMT1 rather work together in both *de novo* methylation and in methylation maintenance (Jeltsch and Jurkowska, 2014). The traditional model of DNA methylation is further challenged by data presented in this thesis.

TET is shown to be far more abundant in the OLs and the MBs of honey bee nurses and foragers than *DNMT3*. This is in line with a study demonstrating much higher *TET* than *DNMT3* levels in whole

brains of nurses and foragers (Wojciechowski et al., 2014). Despite the abundance of *TET*, hydroxymethylated cytosines are only rarely found in the honey bee brain genome. Of the 60 million cytosines in the honey bee genome, approximately 70,000 are methylated in the brain, and only about 14,000 to 22,000 are hydroxymethylated (Lyko et al., 2010; Wojciechowski et al., 2014). This raises the question of the function of high *TET* mRNA levels in the honey bee brain, if not for providing larger amounts of 5hmC. Besides the conversion from 5mC to 5hmC in DNA, mammalian TETs are also involved in recruiting histone modifying complexes (Xu et al., 2012; Pastor et al., 2013). In *Drosophila*, TET promotes 6-adenine methylation and mRNA hydroxymethylation (Zhang et al., 2015; Delatte et al., 2016; Maleszka, 2016). These findings indicate that TET is not restricted to its classical role of DNA cytosine hydroxymethylation, but also performs other reactions. Possibly, the high *TET* mRNA levels in the honey bee brain might implicate a role of TET in RNA modifications in the honey bee.

Another surprising outcome of the thesis is that DNMT3 protein in the honey bee brain is predominantly localized in the cytoplasm, and only rarely found in the nucleus. Given the proposed role of DNMT3 for DNA methylation, this pattern was unexpected. A number of studies in cell cultures and gametocytes report a cytoplasmic and nuclear localization of mammalian DNMT3a, DNMT3b, and DNMTL, with no clear pattern emerging (Lees-Murdock et al., 2005; Zhang et al., 2011; Van Emburgh & Robertson, 2011; Agarwal et al., 2013; Guimarães et al., 2015). Only a few publications investigated the subcellular localization of DNMT3s in brain tissue. One study describes an exclusive nuclear compartmentalization of DNMT3a in post-mitotic brain cells of mice (Feng et al., 2005), which is in line with another report showing a predominant localization of DNMT3a and 3b to the nucleus of post-mitotic neurons of the mouse olfactory epithelium (MacDonald et al., 2005). The role of cytoplasmic DNMT3 is not well understood. In aging mice oocytes, a shifting of the enzyme between the cytoplasm and the nucleus was observed (Zhang et al., 2011). Such a mechanism was proposed to be important for normal development by precisely regulating DNA methylation during specific time windows (Smith and Meissner, 2013). Perhaps, a similar mechanism based on cytoplasmic-nuclear trafficking plays a role for controlling DNA methylation in adult honey bees. In non-dividing brain cells of the honey bee, *de novo* methylation is found rather sporadic, possibly explaining the rare occurrence of nuclear DNMT3 (Lyko et al., 2010). However, a cytoplasmic pool of this enzyme may be important to govern rapid methylation responses to intermittent, but relevant environmental stimuli by a quick shift of DNMTs to the nucleus without the need of lengthy DNMT transcription or translation. Relevant events in the natural behavior of the honey bee which require high and quick molecular flexibility would for example be a sudden switch of tasks if required by the colony, or the transfer of information about profitable food sources into stable long-term memory. The importance of DNMT3 for the formation of long-term memory has been demonstrated before

(Lockett et al., 2010; Biergans et al., 2015). Such events would only be expected to take place in a small number of neurons responsible for the specific memory trace. The occasional nuclear DNMT3 signal in a few nuclei observed in this study may indicate these activity-driven events.

Alternatively, a potential cytoplasmic-nuclear trafficking might be regulated by a circadian rhythm similar to that reported for clock proteins (Meyer, 2006). Honey bee foragers show a rhythmic behavior with active periods during the day and rest at night (Bloch, 2010). This circadian rhythm is associated with the oscillating expression of several hundred genes belonging to the categories development, response to stimuli, muscle contraction, microfilament motor gene expression, generation of precursor metabolites, and energy (Bloch, 2010; Rodriguez-zas et al., 2012). Considering the strong impact of the circadian rhythm on the metabolism, it seems possible that at the end of the work shift of foragers DNMT3 relocates to the nucleus to modify metabolic genes setting the bee to a rest state (Bass, 2012). This notion is supported by the fact that in the honey bee predominantly metabolic genes are subject to methylation (Foret et al., 2012; Ashby et al., 2016). A shift of DNMT3 to the nucleus could also promote the modulation of long-term memory consolidation during the night.

Possibly DNA methylation in the honey bee may even act directly on the circadian clock adjusting it to environmental cues as described for mice (Azzi et al., 2014). Interestingly, *Tim2*, a DEG found to be induced by light exposure in this study, is thought to be responsible for synchronizing the circadian clock to the actual day length in *Drosophila* (Benna et al., 2010). In the present thesis, this gene is differentially methylated between nurses and foragers, speaking for a role of DNA methylation in the circadian rhythm of honey bees.

Finally, the example of TETs being able to hydroxymethylate mRNAs in *Drosophila* suggests that enzymes involved in DNA methylation/demethylation do not necessarily exclusively target DNA as a substrate (Delatte et al., 2016). Modern DNMTs have evolved from RNA-modifying enzymes (Iyer et al., 2011) and thus could still be involved in RNA modification in some organisms. Methylated cytosines have been discovered in all kinds of prokaryotic and eukaryotic RNAs and their formation is mediated by specialized enzymes (Motorin et al., 2010; Cantara et al., 2011; Squires et al., 2012; Popis et al., 2016). However, the cytoplasmic abundance of DNMT3 may indicate a role of this enzyme in RNA methylation in the honey bee.

Conclusion and Outlook

What causes neuronal plasticity? Which forms do exist? How are they mediated? And what are their functional and behavioral consequences? I began the introduction of this thesis raising these fundamental questions on neuronal plasticity and pointed out that numerous advances have been undertaken to answer them. However, many aspects still remain unclear. This is especially true for environmentally-induced plasticity and its underlying molecular mechanisms. Using the honey bee as an innovative model organism, I could identify conserved plasticity-related genes in the context of light-exposure induced structural brain remodeling, thereby introducing them as candidate genes for plasticity in the honey bee. Furthermore, genes, so far not associated with neuronal plasticity, were identified, possibly opening new directions of research on environmentally-induced plasticity. Additionally, the results of my thesis strengthen the hypothesized role of epigenetic mechanisms in neuronal plasticity. However, due to the correlative nature of this study, further experiments are required to prove a role of the introduced molecular mechanisms in neuronal plasticity in the honey bee. Besides adding small pieces to the understanding of brain adaptations to the environment, the results of this thesis also provoke exciting new questions concerning novel regulatory mechanisms of DNMT3, and a possible role of this enzyme beyond DNA methylation. The thesis began with a reference to Ramon y Cajal and I would like to conclude it with a quote of this foresighted scientist that now seems true more than ever.

“The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory.“

Bibliography

Abe K, Chisaka O, van Roy F, Takeichi M. 2004. Stability of dendritic spines and synaptic contacts is controlled by αN-catenin. *Nat Neurosci* 7:357–363.

Ackermann M, Matus A. 2003. Activity-induced targeting of profilin and stabilization of dendritic spine morphology. *Nat Neurosci* 6:1194–1200.

Adewoye AB, Kyriacou CP, Tauber E. 2015. Identification and functional analysis of early gene expression induced by circadian light-resetting in *Drosophila*. *BMC Genomics* 16:570.

Agarwal S, Amin KS, Jagadeesh S, Baishay G, Rao PG, Barua NC, Bhattacharya S, Banerjee PP. 2013. Mahanine restores *RASSF1A* expression by down-regulating DNMT1 and DNMT3B in prostate cancer cells. *Mol Cancer* 12:99.

Agger K, Cloos PAC, Christensen J, Pasini D, Rose S, Rappsilber J, Issaeva I, Canaani E, Salcini AE, Helin K. 2007. UTX and JMJD3 are histone H3K27 demethylases involved in *HOX* gene regulation and development. *Nature* 449:731–734.

Aksoy-Aksel A, Zampa F, Schratt G. 2014. MicroRNAs and synaptic plasticity--a mutual relationship. *Philos Trans R Soc B Biol Sci* 369:20130515–20130515.

Anderson BJ. 2011. Plasticity of Gray Matter Volume: The Cellular and Synaptic Plasticity That Underlies Volumetric Change. *Dev Psychobiol* 53:456–465.

Antonov I, Antonova I, Kandel ER, Hawkins RD. 2003. Activity-Dependent Presynaptic Facilitation and Hebbian LTP Are Both Required and Interact during Classical Conditioning in *Aplysia*. *Neuron* 37:135–147.

Ashby R, Forêt S, Searle I, Maleszka R. 2016. MicroRNAs in Honey Bee Caste Determination. *Sci Rep* 6:18794.

Avarguès-Weber A, Mota T, Giurfa M. 2012. New vistas on honey bee vision. *Apidologie* 43:244–268.

Azzi A, Dallmann R, Casserly A, Rehrauer H, Patrignani A, Maier B, Kramer A, Brown S a. 2014. Circadian behavior is light-reprogrammed by plastic DNA methylation. *Nat Neurosci* 17:377–382.

Bailey CH, Kandel ER. 2008. Chapter 10 Synaptic remodeling, synaptic growth and the storage of long-term memory in *Aplysia*. In: *Progress in Brain Research*. Vol. 169. . p 179–198.

Barth M, Hirsch H V, Meinertzhagen IA, Heisenberg M. 1997. Experience-dependent developmental plasticity in the optic lobe of *Drosophila melanogaster*. *J Neurosci* 17:1493–1504.

Bass J. 2012. Circadian topology of metabolism. *Nature* 491:348–356.

Bastrikova N, Gardner GA, Reece JM, Jeromin A, Dudek SM. 2008. Synapse elimination accompanies functional plasticity in hippocampal neurons. *Proc Natl Acad Sci* 105:3123–3127.

Bayazitov IT, Richardson RJ, Fricke RG, Zakharenko SS. 2007. Slow Presynaptic and Fast Postsynaptic Components of Compound Long-Term Potentiation. *J Neurosci* 27:11510–11521.

Becker N, Wierenga CJ, Fonseca R, Bonhoeffer T, Nägerl UV. 2008. LTD Induction Causes Morphological Changes of Presynaptic Boutons and Reduces Their Contacts with Spines. *Neuron* 60:590–597.

Bednarek E, Caroni P. 2011. β-Adducin Is Required for Stable Assembly of New Synapses and Improved Memory upon Environmental Enrichment. *Neuron* 69:1132–1146.

Behura SK, Whitfield CW. 2010. Correlated expression patterns of microRNA genes with age-dependent behavioural changes in honeybee. *Insect Mol Biol* 19:431–439.

Ben-Moshe Z, Alon S, Mracek P, Faigenbloom L, Tovin A, Vatine GD, Eisenberg E, Foulkes NS, Gothilf Y. 2014. The light-induced transcriptome of the zebrafish pineal gland reveals complex regulation of the circadian clockwork by light. *Nucleic Acids Res* 42:3750–3767.

Benna C, Bonaccorsi S, Wülbeck C, Helfrich-Förster C, Gatti M, Kyriacou CP, Costa R, Sandrelli F. 2010. *Drosophila timeless2* Is Required for Chromosome Stability and Circadian Photoreception. *Curr Biol* 20:346–352.

Bennett EL, Diamond MC, Krech D, Rosenzweig MR. 1964. Chemical and Anatomical Plasticity of Brain. *Science* 146:610–619.

Bernstein BW, Bamburg JR. 2010. ADF/Cofilin: a functional node in cell biology. *Trends Cell Biol* 20:187–195.

Bhutani N, Burns DM, Blau HM. 2011. DNA Demethylation Dynamics. *Cell* 146:866–872.

Biergans SD, Giovanni Galizia C, Reinhard J, Claudianos C. 2015. *Dnmts* and *Tet* target memory-associated genes after appetitive olfactory training in honey bees. *Sci Rep* 5:16223.

Bird A. 2002. DNA methylation patterns and epigenetic memory. *Genes Dev* 16:6–21.

Bloch G. 2010. The Social Clock of the Honeybee. *J Biol Rhythms* 25:307–317.

Bornhauser BC. 2003. MSAP Is a Novel MIR-interacting Protein That Enhances Neurite Outgrowth and Increases Myosin Regulatory Light Chain. *J Biol Chem* 278:35412–35420.

Borrelli E, Nestler EJ, Allis CD, Sassone-Corsi P. 2008. Decoding the Epigenetic Language of Neuronal Plasticity. *Neuron* 60:961–974.

Brill MF, Rosenbaum T, Reus I, Kleineidam CJ, Nawrot MP, Rössler W. 2013. Parallel Processing via a Dual Olfactory Pathway in the Honeybee. *J Neurosci* 33:2443–2456.

Briz V, Zhu G, Wang Y, Liu Y, Avetisyan M, Bi X, Baudry M. 2015. Activity-Dependent Rapid Local RhoA Synthesis Is Required for Hippocampal Synaptic Plasticity. *J Neurosci* 35:2269–2282.

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

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

Campbell G, Shatz CJ. 1992. Synapses formed by identified retinogeniculate axons during the segregation of eye input. *J Neurosci* 12:1847–1858.

Cantara WA, Crain PF, Rozenski J, McCloskey JA, Harris KA, Zhang X, Vendeix FAP, Fabris D, Agris PF. 2011. The RNA modification database, RNAMDB: 2011 update. *Nucleic Acids Res* 39:D195–D201.

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

Chandrasekaran S, Ament SA, Eddy JA, Rodriguez-Zas SL, Schatz BR, Price ND, Robinson GE. 2011. Behavior-specific changes in transcriptional modules lead to distinct and predictable neurogenomic states. *Proc Natl Acad Sci* 108:18020–18025.

Changeux J-P, Danchin A. 1976. Selective stabilisation of developing synapses as a mechanism for the specification of neuronal networks. *Nature* 264:705–712.

Chen T. 2002. A Novel Dnmt3a Isoform Produced from an Alternative Promoter Localizes to Euchromatin and Its Expression Correlates with Active *de Novo* Methylation. *J Biol Chem* 277:38746–38754.

Chernova MN, Jiang L, Friedman DJ, Darman RB, Lohi H, Kere J, Vandorpe DH, Alper SL. 2005. Functional Comparison of Mouse slc26a6 Anion Exchanger with Human SLC26A6 Polypeptide Variants. *J Biol Chem* 280:8564–8580.

Chia PH, Chen B, Li P, Rosen MK, Shen K. 2014. Local F-actin Network Links Synapse Formation and Axon Branching. *Cell* 156:208–220.

Collett TS, Collett M. 2002. Memory use in insect visual navigation. *Nat Rev Neurosci* 3:542–552.

Cowan W, Fawcett J, O'Leary D, Stanfield B. 1984. Regressive events in neurogenesis. *Science* 225:1258–1265.

Cristino AS, Barchuk AR, Freitas FCP, Narayanan RK, Biergans SD, Zhao Z, Simoes ZLP, Reinhard J, Claudianos C. 2014. Neuroligin-associated microRNA-932 targets actin and regulates memory in the honeybee. *Nat Commun* 5:5529.

Crow TJ, Alkon DL. 1980. Associative Behavioral Modification in *Hermissenda*: Cellular Correlates. *Science* 209:412–414.

Dabe EC, Sanford RS, Kohn AB, Bobkova Y, Moroz LL. 2015. DNA Methylation in Basal Metazoans: Insights from Ctenophores. *Integr Comp Biol* 55:1096–1110.

Day JJ, Sweatt JD. 2010. DNA methylation and memory formation. *Nat Neurosci* 13:1319–1323.

Debanne D. 2010. Spike-timing dependent plasticity beyond synapse - pre- and post-synaptic plasticity of intrinsic neuronal excitability. *Front Synaptic Neurosci* 2:21.

Degen J, Kirbach A, Reiter L, Lehmann K, Norton P, Storms M, Koblofsky M, Winter S, Georgieva PB, Nguyen H, et al. 2015. Exploratory behaviour of honeybees during orientation flights. *Anim Behav* 102:45–57.

Delatte B, Wang F, Ngoc LV, Collignon E, Bonvin E, Deplus R, Calonne E, Hassabi B, Putmans P, Awe S, et al. 2016. Transcriptome-wide distribution and function of RNA hydroxymethylcytosine. *Science* 351:282–285.

Dent EW, Callaway JL, Szebenyi G, Baas PW, Kalil K, Szebenyi G, Baas PW, Kalil K. 1999. Reorganization and movement of microtubules in axonal growth cones and developing interstitial branches. *J Neurosci* 19:8894–8908.

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

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

Dyer FC. 1987. Memory and sun compensation by honey bees. *J Comp Physiol A* 160:621–633.

Van Emburgh BO, Robertson KD. 2011. Modulation of Dnmt3b function in vitro by interactions with Dnmt3L, Dnmt3a and Dnmt3b splice variants. *Nucleic Acids Res* 39:4984–5002.

Erber J, Hoormann J, Scheiner R. 2006. Phototactic behaviour correlates with gustatory responsiveness in honey bees (*Apis mellifera* L.). *Behav Brain Res* 174:174–180.

Evgrafov O V, Mersiyanova I, Irobi J, Van Den Bosch L, Dierick I, Leung CL, Schagina O, Verpoorten N, Van Impe K, Fedotov V, et al. 2004. Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. *Nat Genet* 36:602–606.

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

Fahrbach SE. 2006. STRUCTURE OF THE MUSHROOM BODIES OF THE INSECT BRAIN. *Annu Rev Entomol* 51:209–232.

Falckenhayn C, Boerjan B, Raddatz G, Frohme M, Schoofs L, Lyko F. 2013. Characterization of genome methylation patterns in the desert locust *Schistocerca gregaria*. *J Exp Biol* 216:1423–1429.

Farley J. 1988. Associative training results in persistent reductions in a calcium-activated potassium current in *Hermissenda* Type B photoreceptors. *Behav Neurosci* 102:784–802.

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

Feil R, Fraga MF. 2012. Epigenetics and the environment: emerging patterns and implications. *Nat Rev Genet* 13:97–109.

Feng J, Chang H, Li E, Fan G. 2005. Dynamic expression of de novo DNA methyltransferases Dnmt3a and Dnmt3b in the central nervous system. *J Neurosci Res* 79:734–746.

Feng J, Zhou Y, Campbell SL, Le T, Li E, Sweatt JD, Silva AJ, Fan G. 2010. Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nat Neurosci* 13:423–430.

Flavell SW, Greenberg ME. 2008. Signaling Mechanisms Linking Neuronal Activity to Gene Expression and Plasticity of the Nervous System. *Annu Rev Neurosci* 31:563–590.

Fleischmann A, Hvalby O, Jensen V, Strekalova T, Zacher C, Layer LE, Kvello A, Reschke M, Spanagel R, Sprengel R, et al. 2003. Impaired long-term memory and NR2A-type NMDA receptor-dependent synaptic plasticity in mice lacking c-Fos in the CNS. *J Neurosci* 23:9116–9122.

Foret S, Kucharski R, Pellegrini M, Feng S, Jacobsen SE, Robinson GE, Maleszka R. 2012. DNA methylation dynamics, metabolic fluxes, gene splicing, and alternative phenotypes in honey bees. *Proc Natl Acad Sci* 109:4968–4973.

Frantz MG, Kast RJ, Dorton HM, Chapman KS, McGee AW. 2016. Nogo Receptor 1 Limits Ocular Dominance Plasticity but not Turnover of Axonal Boutons in a Model of Amblyopia. *Cereb Cortex* 26:1975–1985.

Friedrich A. 2004. Learning at Different Satiation Levels Reveals Parallel Functions for the cAMP-Protein Kinase A Cascade in Formation of Long-Term Memory. *J Neurosci* 24:4460–4468.

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

Fu X, Brown KJ, Yap CC, Winckler B, Jaiswal JK, Liu JS. 2013. Doublecortin (Dcx) Family Proteins Regulate Filamentous Actin Structure in Developing Neurons. *J Neurosci* 33:709–721.

Galimberti I, Gogolla N, Alberi S, Santos AF, Muller D, Caroni P. 2006. Long-Term Rearrangements of Hippocampal Mossy Fiber Terminal Connectivity in the Adult Regulated by Experience. *Neuron* 50:749–763.

Gehring KB, Heufelder K, Feige J, Bauer P, Dyck Y, Ehrhardt L, Kühnemund J, Bergmann A, Göbel J, Isecke M, et al. 2016. Involvement of phosphorylated *Apis mellifera* CREB in gating a honeybee's behavioral response to an external stimulus. *Learn Mem* 23:195–207.

Geinisman Y, Berry RW, Disterhoft JF, Power JM, Van der Zee E a. 2001. Associative learning elicits the formation of multiple-synapse boutons. *J Neurosci* 21:5568–5573.

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

Greenough WT, Black JE, Wallace CS. 1987. Experience and brain development. *Child Dev*:539–559.

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

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

Grünbaum L, Müller U. 1998. Induction of a Specific Olfactory Memory Leads to a Long-Lasting Activation of Protein Kinase C in the Antennal Lobe of the Honeybee. *J Neurosci* 18:4384–4392.

Guimarães DM, Antunes DM, Duarte CME, Ferro LB, Nunes FD. 2015. DNA methyltransferase immunohistochemical expression in odontogenic tumours. *J Oral Pathol Med* 44:59–66.

Guo JU, Ma DK, Mo H, Ball MP, Jang M-H, Bonaguidi MA, Balazer JA, Eaves HL, Xie B, Ford E, et al. 2011. Neuronal activity modifies the DNA methylation landscape in the adult brain. *Nat Neurosci* 14:1345–1351.

Guo X, Wang L, Li J, Ding Z, Xiao J, Yin X, He S, Shi P, Dong L, Li G, et al. 2014. Structural insight into autoinhibition and histone H3-induced activation of DNMT3A. *Nature* 517:640–644.

Guzman-Karlsson MC, Meadows JP, Gavin CF, Hablitz JJ, Sweatt JD. 2014. Transcriptional and epigenetic regulation of Hebbian and non-Hebbian plasticity. *Neuropharmacology* 80:3–17.

Heisenberg M, Heusipp M, Wanke C. 1995. Structural plasticity in the *Drosophila* brain. *J Neurosci* 15:1951–1960.

Hensch TK. 2005. Critical period plasticity in local cortical circuits. *Nat Rev Neurosci* 6:877–888.

Herb BR, Wolschin F, Hansen KD, Aryee MJ, Langmead B, Irizarry R, Amdam G V, Feinberg AP. 2012. Reversible switching between epigenetic states in honeybee behavioral subcastes. *Nat Neurosci* 15:1371–1373.

Hertel H. 1983. Change of synapse frequency in certain photoreceptors of the honeybee after chromatic deprivation. *J Comp Physiol A* 151:477–482.

Holahan MR, Rekart JL, Sandoval J, Routtenberg A. 2006. Spatial learning induces presynaptic structural remodeling in the hippocampal mossy fiber system of two rat strains. *Hippocampus* 16:560–570.

Hölldobler B, Wilson EO. 2009. The superorganism: the beauty, elegance, and strangeness of insect societies. *Norton & Company*.

Holtmaat A, Svoboda K. 2009. Experience-dependent structural synaptic plasticity in the mammalian brain. *Nat Rev Neurosci* 10:647–658.

Holtmaat A, Wilbrecht L, Knott GW, Welker E, Svoboda K. 2006. Experience-dependent and cell-type-specific spine growth in the neocortex. *Nature* 441:979–983.

Hourcade B, Muenz TS, Sandoz JC, Rössler W, Devaud JM. 2010. Long-Term Memory Leads to Synaptic Reorganization in the Mushroom Bodies: A Memory Trace in the Insect Brain? *J Neurosci* 30:6461–6465.

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

Hu J, Bai X, Bowen JR, Dolat L, Korobova F, Yu W, Baas PW, Svitkina T, Gallo G, Spiliotis ET. 2012. Septin-Driven Coordination of Actin and Microtubule Remodeling Regulates the Collateral Branching of Axons. *Curr Biol* 22:1109–1115.

Hu J-L, Zhou BO, Zhang R-R, Zhang K-L, Zhou J-Q, Xu G-L. 2009. The N-terminus of histone H3 is required for de novo DNA methylation in chromatin. *Proc Natl Acad Sci* 106:22187–22192.

Hu Y-S, Long N, Pigino G, Brady ST, Lazarov O. 2013. Molecular Mechanisms of Environmental Enrichment: Impairments in Akt/GSK3β, Neurotrophin-3 and CREB Signaling. *PLoS One* 8:e64460.

Impey S, Davare M, Lasiek A, Fortin D, Ando H, Varlamova O, Obrietan K, Soderling TR, Goodman RH, Wayman GA. 2010. An activity-induced microRNA controls dendritic spine formation by regulating Rac1-PAK signaling. *Mol Cell Neurosci* 43:146–156.

Inano K, Suetake I, Ueda T, Miyake Y, Nakamura M, Okada M, Tajima S. 2000. Maintenance-type DNA methyltransferase is highly expressed in post-mitotic neurons and localized in the cytoplasmic compartment. *J Biochem* 128:315–321.

Ito K, Shinomiya K, Ito M, Armstrong JD, Boyan G, Hartenstein V, Harzsch S, Heisenberg M, Homberg U, Jenett A, et al. 2014. A Systematic Nomenclature for the Insect Brain. *Neuron* 81:755–765.

Iyer LM, Abhiman S, Aravind L. 2011. Natural History of Eukaryotic DNA Methylation Systems. In: *Progress in molecular biology and translational science*. Vol. 101. . p 25–104.

Jeltsch A, Jurkowska RZ. 2014. New concepts in DNA methylation. *Trends Biochem Sci* 39:310–318.

Jones PA. 2012. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 13:484–492.

Jurkowska RZ, Jurkowski TP, Jeltsch A. 2011a. Structure and Function of Mammalian DNA Methyltransferases. *ChemBioChem* 12:206–222.

Jurkowska RZ, Rajavelu A, Anspach N, Urbanke C, Jankevicius G, Ragozin S, Nellen W, Jeltsch A. 2011b. Oligomerization and Binding of the Dnmt3a DNA Methyltransferase to Parallel DNA Molecules. *J Biol Chem* 286:24200–24207.

Kalil K, Dent EW. 2013. Branch management: mechanisms of axon branching in the developing vertebrate CNS. *Nat Rev Neurosci* 15:7–18.

Kim IH, Park SK, Hong ST, Jo YS, Kim EJ, Park EH, Han SB, Shin H-S, Sun W, Kim HT, et al. 2009. Inositol 1,4,5-Trisphosphate 3-Kinase A Functions As a Scaffold for Synaptic Rac Signaling. *J Neurosci* 29:14039–14049.

Kim IH, Park SK, Sun W, Kang Y, Kim HT, Kim H. 2004. Spatial learning enhances the expression of inositol 1,4,5-trisphosphate 3-kinase A in the hippocampal formation of rat. *Mol Brain Res* 124:12–19.

Kim IH, Racz B, Wang H, Burianek L, Weinberg R, Yasuda R, Wetsel WC, Soderling SH. 2013. Disruption of Arp2/3 Results in Asymmetric Structural Plasticity of Dendritic Spines and Progressive Synaptic and Behavioral Abnormalities. *J Neurosci* 33:6081–6092.

Kim M, Jeong Y, Chang Y-C. 2015. Extracellular matrix protein reelin regulate dendritic spine density through CaMKIIβ. *Neurosci Lett* 599:97–101.

Kiorpes L. 2015. Visual development in primates: Neural mechanisms and critical periods. *Dev Neurobiol* 75:1080–1090.

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

Kleim J a, Swain R a, Armstrong K a, Napper RMA, Jones T a, Greenough WT. 1998. Selective Synaptic Plasticity within the Cerebellar Cortex Following Complex Motor Skill Learning. *Neurobiol Learn Mem* 69:274–289.

Knott GW, Holtmaat A, Wilbrecht L, Welker E, Svoboda K. 2006. Spine growth precedes synapse formation in the adult neocortex in vivo. *Nat Neurosci* 9:1117–1124.

Kolb B, Cioe J, Comeau W. 2008. Contrasting effects of motor and visual spatial learning tasks on dendritic arborization and spine density in rats. *Neurobiol Learn Mem* 90:295–300.

Kolb B, Gibb R. 2014. Searching for the principles of brain plasticity and behavior. *Cortex* 58:251–260.

Kolb B, Whishaw IQ. 1998. BRAIN PLASTICITY AND BEHAVIOR. *Annu Rev Psychol* 49:43–64.

Kolb B. 1998. Age, Experience and the Changing Brain. *Neurosci Biobehav Rev* 22:143–159.

Kucharski R, Foret S, Maleszka R. 2015. EGFR gene methylation is not involved in Royalactin controlled phenotypic polymorphism in honey bees. *Sci Rep* 5:14070.

Kucharski R, Maleszka J, Foret S, Maleszka R. 2008. Nutritional Control of Reproductive Status in Honeybees via DNA Methylation. *Science* 319:1827–1830.

Kucharski R, Maleszka R. 2002a. Evaluation of differential gene expression during behavioral development in the honeybee using microarrays and northern blots. *Genome Biol* 3:RESEARCH 0007.–0007.9.

Kucharski R, Maleszka R. 2002b. Molecular profiling of behavioural development: differential expression of mRNAs for inositol 1,4,5-trisphosphate 3-kinase isoforms in naive and experienced honeybees (*Apis mellifera*). *Mol brain Res* 99:92–101.

Lamprecht R, Farb CR, Rodrigues SM, LeDoux JE. 2006. Fear conditioning drives profilin into amygdala dendritic spines. *Nat Neurosci* 9:481–483.

Law JA, Jacobsen SE. 2010. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet* 11:204–220.

Law R, Dixon-Salazar T, Jerber J, Cai N, Abbasi AA, Zaki MS, Mittal K, Gabriel SB, Rafiq MA, Khan V, et al. 2014. Biallelic Truncating Mutations in *FMN2*, Encoding the Actin-Regulatory Protein Formin 2, Cause Nonsyndromic Autosomal-Recessive Intellectual Disability. *Am J Hum Genet* 95:721–728.

Lee JD. 2002. The ubiquitin ligase Hyperplastic discs negatively regulates hedgehog and decapentaplegic expression by independent mechanisms. *Development* 129:5697–5706.

Lees-Murdock DJ, Shovlin TC, Gardiner T, De Felici M, Walsh CP. 2005. DNA methyltransferase expression in the mouse germ line during periods of de novo methylation. *Dev Dyn* 232:992–1002.

Li-Byarlay H, Li Y, Stroud H, Feng S, Newman TC, Kaneda M, Hou KK, Worley KC, Elsik CG, Wickline SA, et al. 2013. RNA interference knockdown of *DNA methyl-transferase 3* affects gene alternative splicing in the honey bee. *Proc Natl Acad Sci* 110:12750–12755.

Lim Y, Traber MG. 2007. Alpha-Tocopherol Transfer Protein (α-TTP): Insights from Alpha-Tocopherol Transfer Protein Knockout Mice. *Nutr Res Pract* 1:247–253.

Lin Y-C, Hsieh L-C, Kuo M-W, Yu J, Kuo H-H, Lo W-L, Lin R-J, Yu AL, Li W-H. 2007. Human *TRIM71* and Its Nematode Homologue Are Targets of *let-7* MicroRNA and Its Zebrafish Orthologue Is Essential for Development. *Mol Biol Evol* 24:2525–2534.

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

Lindauer M. 1961. Communication among social bees. *Cambridge, Massachusetts, USA, Harvard University Press*.

Lisman J, Yasuda R, Raghavachari S. 2012. Mechanisms of CaMKII action in long-term potentiation. *Nat Rev Neurosci* 13:169–182.

Lockett GA, Helliwell P, Maleszka R. 2010. Involvement of DNA methylation in memory processing in the honey bee. *Neuroreport* 21:812–816.

Lockett GA, Kucharski R, Maleszka R. 2012. DNA methylation changes elicited by social stimuli in the brains of worker honey bees. *Genes, Brain Behav* 11:235–242.

Lockett GA, Wilkes F, Helliwell P, Maleszka R. 2014. Contrasting Effects of Histone Deacetylase Inhibitors on Reward and Aversive Olfactory Memories in the Honey Bee. *Insects* 5:377–398.

Lourenco AP, Mackert A, Cristino A dos S, Simoes ZLP, Lourenço AP, Mackert A, dos Santos Cristino A, Simões ZLP. 2008. Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera*, by quantitative real-time RT-PCR. *Apidologie* 39:372–385.

Lutz CC, Rodriguez-Zas SL, Fahrbach SE, Robinson GE. 2012. Transcriptional response to foraging experience in the honey bee mushroom bodies. *Dev Neurobiol* 72:153–166.

Lyko F, Foret S, Kucharski R, Wolf S, Falckenhayn C, Maleszka R. 2010. The honey bee epigenomes: differential methylation of brain DNA in queens and workers. *PLoS Biol* 8:e1000506.

Lyko F, Maleszka R. 2011. Insects as innovative models for functional studies of DNA methylation. *Trends Genet* 27:127–131.

MacDonald JL, Gin CSY, Roskams AJ. 2005. Stage-specific induction of DNA methyltransferases in olfactory receptor neuron development. *Dev Biol* 288:461–473.

Malenka RC, Bear MF. 2004. LTP and LTD. *Neuron* 44:5–21.

Maleszka J, Barron AB, Helliwell PG, Maleszka R. 2009. Effect of age, behaviour and social environment on honey bee brain plasticity. *J Comp Physiol A* 195:733–740.

Maleszka R. 2008. Epigenetic integration of environmental and genomic signals in honey bees: the critical interplay of nutritional, brain and reproductive networks. *Epigenetics* 3:188–192.

Maleszka R. 2014. The social honey bee in biomedical research: realities and expectations. *Drug Discov Today Dis Model* 12:7–13.

Maleszka R. 2016. Epigenetic code and insect behavioural plasticity. *Curr Opin Insect Sci* 15:45–52.

Matsuzaki M, Honkura N, Ellis-Davies GCR, Kasai H. 2004. Structural basis of long-term potentiation in single dendritic spines. *Nature* 429:761–766.

Meadows JP, Guzman-Karlsson MC, Phillips S, Holleman C, Posey JL, Day JJ, Hablitz JJ, Sweatt JD. 2015. DNA methylation regulates neuronal glutamatergic synaptic scaling. *Sci Signal* 8:ra61–ra61.

Meng Y, Zhang Y, Tregoubov V, Janus C, Cruz L, Jackson M, Lu W-Y, MacDonald JF, Wang JY, Falls DL, et al. 2002. Abnormal Spine Morphology and Enhanced LTP in LIMK-1 Knockout Mice. *Neuron* 35:121–133.

Menzel R, Fuchs J, Nadler L, Weiss B, Kumbischinski N, Adebiyi D, Hartfil S, Greggers U. 2010. Dominance of the odometer over serial landmark learning in honeybee navigation. *Naturwissenschaften* 97:763–767.

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

Menzel R. 2012. The honeybee as a model for understanding the basis of cognition. *Nat Rev Neurosci* 13:758–768.

Merschbaecher K, Haettig J, Mueller U. 2012. Acetylation-Mediated Suppression of Transcription-Independent Memory: Bidirectional Modulation of Memory by Acetylation. *PLoS One* 7:e45131.

Merschbaecher K, Hatko L, Folz J, Mueller U. 2016. Inhibition of different histone acetyltransferases (HATs) uncovers transcription-dependent and -independent acetylation-mediated mechanisms in memory formation. *Learn Mem* 23:83–89.

Messaoudi E, Kanhema T, Soule J, Tiron A, Dagyte G, da Silva B, Bramham CR. 2007. Sustained Arc/Arg3.1 Synthesis Controls Long-Term Potentiation Consolidation through Regulation of Local Actin Polymerization in the Dentate Gyrus In Vivo. *J Neurosci* 27:10445–10455.

Meyer D, Bonhoeffer T, Scheuss V. 2014. Balance and Stability of Synaptic Structures during Synaptic Plasticity. *Neuron* 82:430–443.

Meyer P. 2006. PER-TIM Interactions in Living *Drosophila* Cells: An Interval Timer for the Circadian Clock. *Science* 311:226–229.

Miller CA, Sweatt JD. 2007. Covalent Modification of DNA Regulates Memory Formation. *Neuron* 53:857–869.

Min K. 1999. Preventing Neurodegeneration in the *Drosophila* Mutant bubblegum. *Science* 284:1985–1988.

Moore LD, Le T, Fan G. 2013. DNA Methylation and Its Basic Function. *Neuropsychopharmacology* 38:23–38.

Motorin Y, Lyko F, Helm M. 2010. 5-methylcytosine in RNA: detection, enzymatic formation and biological functions. *Nucleic Acids Res* 38:1415–1430.

Mower AF, Liao DS, Nestler EJ, Neve RL, Ramoa AS. 2002. cAMP/Ca2+ response element-binding protein function is essential for ocular dominance plasticity. *J Neurosci* 22:2237–2245.

Mozzachiodi R, Byrne JH. 2010. More than synaptic plasticity: role of nonsynaptic plasticity in learning and memory. *Trends Neurosci* 33:17–26.

Muenz TS, Groh C, Maisonnasse A, Le Conte Y, Plettner E, Rössler W. 2015. Neuronal plasticity in the mushroom body calyx during adult maturation in the honeybee and possible pheromonal influences. *Dev Neurobiol* 75:1368–1384.

Murakoshi H, Wang H, Yasuda R. 2011. Local, persistent activation of Rho GTPases during plasticity of single dendritic spines. *Nature* 472:100–104.

Nägerl UV, Eberhorn N, Cambridge SB, Bonhoeffer T. 2004. Bidirectional Activity-Dependent Morphological Plasticity in Hippocampal Neurons. *Neuron* 44:759–767.

Nakadate K, Imamura K, Watanabe Y. 2013. c-Fos activity mapping reveals differential effects of noradrenaline and serotonin depletion on the regulation of ocular dominance plasticity in rats. *Neuroscience* 235:1–9.

Ohnami S, Endo M, Hirai S, Uesaka N, Hatanaka Y, Yamashita T, Yamamoto N. 2008. Role of RhoA in Activity-Dependent Cortical Axon Branching. *J Neurosci* 28:9117–9121.

Okano M, Bell DW, Haber DA, Li E. 1999. DNA Methyltransferases Dnmt3a and Dnmt3b Are Essential for De Novo Methylation and Mammalian Development. *Cell* 99:247–257.

Ozawa S, Kamiya H, Tsuzuki K. 1998. Glutamate receptors in the mammalian central nervous system. *Prog Neurobiol* 54:581–618.

De Paola V, Holtmaat A, Knott G, Song S, Wilbrecht L, Caroni P, Svoboda K. 2006. Cell Type-Specific Structural Plasticity of Axonal Branches and Boutons in the Adult Neocortex. *Neuron* 49:861–875.

Pastor WA, Aravind L, Rao A. 2013. TETonic shift: biological roles of TET proteins in DNA demethylation and transcription. *Nat Rev Mol Cell Biol* 14:341–356.

Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30:e36.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.

Popis MC, Blanco S, Frye M. 2016. Posttranscriptional methylation of transfer and ribosomal RNA in stress response pathways, cell differentiation, and cancer. *Curr Opin Oncol* 28:65–71.

Qin Q-H, Wang Z-L, Tian L-Q, Gan H-Y, Zhang S-W, Zeng Z-J. 2014. The integrative analysis of microRNA and mRNA expression in *Apis mellifera* following maze-based visual pattern learning. *Insect Sci* 21:619–636.

Qin W, Wolf P, Liu N, Link S, Smets M, Mastra F La, Forné I, Pichler G, Hörl D, Fellinger K, et al. 2015. DNA methylation requires a DNMT1 ubiquitin interacting motif (UIM) and histone ubiquitination. *Cell Res* 25:911–929.

Qiu Z, Ghosh A. 2008. A Calcium-Dependent Switch in a CREST-BRG1 Complex Regulates Activity-Dependent Gene Expression. *Neuron* 60:775–787.

Reim T, Thamm M, Rolke D, Blenau W, Scheiner R. 2012. Suitability of three common reference genes for quantitative real-time PCR in honey bees. *Apidologie* 44:342–350.

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

Robinson GE. 1992. Regulation of Division of Labor in Insect Societies. *Annu Rev Entomol* 37:637–665.

Rodriguez-zas SL, Southey BR, Shemesh Y, Rubin EB, Cohen M, Robinson GE, Bloch G. 2012. Microarray analysis of natural socially regulated plasticity in circadian rhythms of honey bees. *J Biol Rhythms* 27:12–24.

Rosenzweig MR, Krech D, Bennett EL, Zolman JF. 1962. Variation in environmental complexity and brain measures. *J Comp Physiol Psychol* 55:1092.

Rössler W, Groh C. 2012. Plasticity of synaptic microcircuits in the mushroom-body calyx of the honey bee. In: *Honeybee neurobiology and behavior—a tribute to Randolf Menzel*. *Springer Netherlands*. p 141–153.

Ryan B, Joilin G, Williams JM. 2015. Plasticity-related microRNA and their potential contribution to the maintenance of long-term potentiation. *Front Mol Neurosci* 8.

Rybak J, Meinertzhagen IA. 1997. The effects of light reversals on photoreceptor synaptogenesis in the fly *Musca domestica*. *Eur J Neurosci* 9:319–333.

Sanchez-Carron G, Garcia-Garcia MI, Lopez-Rodriguez AB, Jimenez-Garcia S, Sola-Carvajal A, Garcia-Carmona F, Sanchez-Ferrer A. 2011. Molecular Characterization of a Novel N-Acetylneuraminate Lyase from *Lactobacillus plantarum* WCFS1. *Appl Environ Microbiol* 77:2471–2478.

Sanyal S, Sandstrom DJ, Hoeffer C a, Ramaswami M. 2002. AP-1 functions upstream of CREB to control synaptic plasticity in *Drosophila*. *Nature* 416:870–874.

Sasaki K, Adachi S, Yamamoto T, Murakami T, Tanaka K, Takahashi M. 1988. Effects of denaturation with HCl on the immunological staining of bromodeoxyuridine incorporated into DNA. *Cytometry* 9:93–96.

Scholl C, Kübert N, Muenz TS, Ro ssler W. 2015. CaMKII knockdown affects both early and late phases of olfactory long-term memory in the honeybee. *J Exp Biol* 218:3788–3796.

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

Schulz DJ, Huang Z, Robinson GE. 1998. Effects of colony food shortage on behavioral development in honey bees. *Behav Ecol Sociobiol* 42:295–303.

Sin WC, Haas K, Ruthazer ES, Cline HT. 2002. Dendrite growth increased by visual activity requires NMDA receptor and Rho GTPases. *Nature* 419:475–480.

Smalheiser NR. 2014. The RNA-centred view of the synapse: non-coding RNAs and synaptic plasticity. *Philos Trans R Soc B Biol Sci* 369:20130504–20130504.

Smith ZD, Meissner A. 2013. DNA methylation: roles in mammalian development. *Nat Rev Genet* 14:204–220.

Snell-Rood EC. 2013. An overview of the evolutionary causes and consequences of behavioural plasticity. *Anim Behav* 85:1004–1011.

Spence EF, Soderling SH. 2015. Actin Out: Regulation of the Synaptic Cytoskeleton. *J Biol Chem* 290:28613–28622.

Squires JE, Patel HR, Nousch M, Sibbritt T, Humphreys DT, Parker BJ, Suter CM, Preiss T. 2012. Widespread occurrence of 5-methylcytosine in human coding and non-coding RNA. *Nucleic Acids Res* 40:5023–5033.

Steinberg SJ, Morgenthaler J, Heinzer AK, Smith KD, Watkins PA. 2000. Very Long-chain Acyl-CoA Synthetases: HUMAN "BUBBLEGUM" REPRESENTS A NEW FAMILY OF PROTEINS CAPABLE OF ACTIVATING VERY LONG-CHAIN FATTY ACIDS. *J Biol Chem* 275:35162–35169.

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

Strahl BD, Allis CD. 2000. The language of covalent histone modifications. *Nature* 403:41–45.

Strube-Bloss MF, Nawrot MP, Menzel R. 2011. Mushroom Body Output Neurons Encode Odor-Reward Associations. *J Neurosci* 31:3129–3140.

Sui L, Wang Y, Ju L, Chen M. 2012. Epigenetic regulation of *reelin* and genes in long-term potentiation in rat medial prefrontal cortex. *Neurobiol Learn Mem* 97:425–440.

Sutton MA, Schuman EM. 2006. Dendritic Protein Synthesis, Synaptic Plasticity, and Memory. *Cell* 127:49–58.

Szenker E, Ray-Gallet D, Almouzni G. 2011. The double face of the histone variant H3.3. *Cell Res* 21:421–434.

Tagawa Y, Kanold PO, Majdan M, Shatz CJ. 2005. Multiple periods of functional ocular dominance plasticity in mouse visual cortex. *Nat Neurosci* 8:380–388.

Takahashi H, McCaffery JM, Irizarry RA, Boeke JD. 2006. Nucleocytosolic Acetyl-Coenzyme A Synthetase Is Required for Histone Acetylation and Global Transcription. *Mol Cell* 23:207–217.

Thamm M, Balfanz S, Scheiner R, Baumann A, Blenau W. 2010. Characterization of the 5-HT1A receptor of the honeybee (*Apis mellifera*) and involvement of serotonin in phototactic behavior. *Cell Mol Life Sci* 67:2467–2479.

Wang Y, Jorda M, Jones PL, Maleszka R, Ling X, Robertson HM, Mizzen CA, Peinado MA, Robinson GE. 2006. Functional CpG Methylation System in a Social Insect. *Science* 314:645–647.

Wayman GA, Impey S, Marks D, Saneyoshi T, Grant WF, Derkach V, Soderling TR. 2006. Activity-Dependent Dendritic Arborization Mediated by CaM-Kinase I Activation and Enhanced CREB-Dependent Transcription of Wnt-2. *Neuron* 50:897–909.

Wedd L, Kucharski R, Maleszka R. 2016. Differentially methylated obligatory epialleles modulate context-dependent *LAM* gene expression in the honeybee *Apis mellifera*. *Epigenetics* 11:1–10.

Weger BD, Sahinbas M, Otto GW, Mracek P, Armant O, Dolle D, Lahiri K, Vallone D, Ettwiller L, Geisler R, et al. 2011. The Light Responsive Transcriptome of the Zebrafish: Function and Regulation. *PLoS One* 6:e17080.

Weinstock GM, Robinson GE, Gibbs RA, Worley KC, Evans JD, Maleszka R, Robertson HM, Weaver DB, Beye M, Bork P, et al. 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* 443:931–949.

West AE, Greenberg ME. 2011. Neuronal Activity-Regulated Gene Transcription in Synapse Development and Cognitive Function. *Cold Spring Harb Perspect Biol* 3:a005744–a005744.

Wettstein G, Bellaye PS, Micheau O, Bonniaud P. 2012. Small heat shock proteins and the cytoskeleton: An essential interplay for cell integrity? *Int J Biochem Cell Biol* 44:1680–1686.

Whitfield CW, Ben-Shahar Y, Brillet C, Leoncini I, Crauser D, LeConte Y, Rodriguez-Zas S, Robinson GE. 2006. Genomic dissection of behavioral maturation in the honey bee. *Proc Natl Acad Sci* 103:16068–16075.

Whitfield CW. 2003. Gene Expression Profiles in the Brain Predict Behavior in Individual Honey Bees. *Science* 302:296–299.

Wiesel TN, Hubel DH. 1963. SINGLE-CELL RESPONSES IN STRIATE CORTEX OF KITTENS DEPRIVED OF VISION IN ONE EYE. *J Neurophysiol* 26:1003–1017.

Wijnen H, Naef F, Boothroyd C, Claridge-Chang A, Young MW. 2006. Control of Daily Transcript Oscillations in *Drosophila* by Light and the Circadian Clock. *PLoS Genet* 2:e39.

Williams KL, Mearow KM. 2011. Phosphorylation status of heat shock protein 27 influences neurite growth in adult dorsal root ganglion sensory neurons in vitro. *J Neurosci Res* 89:1160–1172.

Wilson EO. 1971. The insect societies. *Cambridge, Massachusetts, USA, Harvard University Press*.

Windhorst S, Minge D, Bähring R, Hüser S, Schob C, Blechner C, Lin H-Y, Mayr GW, Kindler S. 2012. Inositol-1,4,5-trisphosphate 3-kinase A regulates dendritic morphology and shapes synaptic Ca2+ transients. *Cell Signal* 24:750–757.

Winston ML. 1991. The Biology of the Honey Bee. *Cambridge, Massachusetts, USA, Harvard University Press*.

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

Wojciechowski M, Rafalski D, Kucharski R, Misztal K, Maleszka J, Bochtler M, Maleszka R. 2014. Insights into DNA hydroxymethylation in the honeybee from in-depth analyses of TET dioxygenase. *Open Biol* 4:140110–140110.

Xu T, Yu X, Perlik AJ, Tobin WF, Zweig JA, Tennant K, Jones T, Zuo Y. 2009. Rapid formation and selective stabilization of synapses for enduring motor memories. *Nature* 462:915–919.

Xu Y, Xu C, Kato A, Tempel W, Abreu JG, Bian C, Hu Y, Hu D, Zhao B, Cerovina T, et al. 2012. Tet3 CXXC Domain and Dioxygenase Activity Cooperatively Regulate Key Genes for *Xenopus* Eye and Neural Development. *Cell* 151:1200–1213.

y Cajal SR. 1928. Degeneration and regeneration of the nervous system. Volume 1. London: *Oxford University Press*.

Yang N, Higuchi O, Ohashi K, Nagata K, Wada A, Kangawa K, Nishida E, Mizuno K. 1998. Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* 393:809–812.

Yilmaz A, Lindenberg A, Albert S, Grübel K, Spaethe J, Rössler W, Groh C. 2016. Age-related and light-induced plasticity in opsin gene expression and in primary and secondary visual centers of the nectar-feeding ant *Camponotus rufipes*. *Dev Neurobiol*.

Yuan Q, Xiang Y, Yan Z, Han C, Jan LY, Jan YN. 2011. Light-Induced Structural and Functional Plasticity in *Drosophila* Larval Visual System. *Science* 333:1458–1462.

Zayed A, Robinson GE. 2012. Understanding the Relationship Between Brain Gene Expression and Social Behavior: Lessons from the Honey Bee. *Annu Rev Genet* 46:591–615.

Zhang G, Huang H, Liu D, Cheng Y, Liu X, Zhang W, Yin R, Zhang D, Zhang P, Liu J, et al. 2015. N6-Methyladenine DNA Modification in *Drosophila*. *Cell* 161:893–906.

Zhang L, Lu D-Y, Ma W-Y, Li Y. 2011. Age-related changes in the localization of DNA methyltransferases during meiotic maturation in mouse oocytes. *Fertil Steril* 95:1531–1534.e1.

Zhang Y, Jurkowska R, Soeroes S, Rajavelu A, Dhayalan A, Bock I, Rathert P, Brandt O, Reinhardt R, Fischle W, et al. 2010. Chromatin methylation activity of Dnmt3a and Dnmt3a/3L is guided by interaction of the ADD domain with the histone H3 tail. *Nucleic Acids Res* 38:4246–4253.

Zilles K. 1992. Neuronal plasticity as an adaptive property of the central nervous system. *Ann Anat* 174:383–391.

Zou Y, Chiu H, Zinovyeva A, Ambros V, Chuang C-F, Chang C. 2013. Developmental Decline in Neuronal Regeneration by the Progressive Change of Two Intrinsic Timers. *Science* 340:372–376.

Publications List

Becker N, Kucharski R, Rössler W, Maleszka R. 2016. Age-dependent Transcriptional and Epigenomic Responses to Light Exposure in the Honey Bee Brain. *FEBS Open Bio*. DOI: 10.1002/2211-5463.12084

Weißflog L, **Becker N**, Bossert N, Freudenberg F, Kittel-Schneider S, Reif A. 2016. Expressional profile of the diacylglycerol kinase eta gene DGKH. *Eur Arch Psychiatry Clin Neurosci*:1–10.

Danksagung und Acknowledgements

Meine Doktorarbeit mit viel Spaß, Freude und Begeisterung zu Ende gebracht zu haben wäre nicht ohne die Hilfe von Kollegen, Freunden und Familie möglich gewesen. Ihnen allen gebührt mein herzlichster Dank.

An oberster Stelle ist mein Doktorvater Wolfgang zu nennen. Ich hatte das Privileg mit viel Freiheit meinen Forschungsinteressen nachgehen zu können, was mich über die dreieinhalb Jahre extrem motiviert hielt. Trotzdem durfte ich eine enge Betreuung genießen, bei der Wolfgang jederzeit bereit war neue Ideen zu diskutieren oder mit hilfreichen Rat zur Seite zu stehen. Besonders möchte ich mich bei Wolfgangs für seinen Einsatz bedanken, dass er mir das Knüpfen von Kontakten oder Eingehen von Kooperation mit anderen Wissenschaftlern ermöglicht hat. So hatte ich die großartige Gelegenheit auf viele Fachkongresse in ferne oder unbekannte Länder wie Uruguay und Rauischholzhausen zu reisen und wertvolle und bereichernde Kontakte zu anderen Wissenschaftlern herzustellen.

Bei Christian, meinem Zweitbetreuer, möchte ich mich speziell dafür bedanken, dass er meine Arbeit nicht nur von Beginn an mit großem Interesse verfolgt, sondern auch mit konstruktiven Kommentaren und praktischer Hilfe vorangetrieben hat. Ich war froh zu wissen, irgendwo im Keller der EM, versteckt zwischen verschiedensten Mikroskopen der letzten Jahrhunderte, einen so engagierten und kompetenten Ansprechpartner für meine Anliegen anzutreffen.

It was a great fortune to recruit Ryszard as my third supervisor for the thesis. I will not forget his and Joanna's generous hospitality during my visits in Ryszard's lab. That, thanks to them, I had a wonderful and exciting time during my stays in the capital of Australia, although not Sydney or Melbourne but Canberra, speaks for itself. I profited a lot from Ryszard's knowledge and expertise which he was gladly willing to share. Email requests were always quickly answered, mostly even in the middle of the night, proving Ryszard's great dedication. My thanks certainly also go to all members of Ryszard's Lab. Joanna and Robert were wonderful in patiently teaching me new techniques and helping me with my experiments, Sylvain was very supportive in analyzing data, and Paul took care that I always had enough bees and coffee. Paul secretly brews the best coffee of Canberra.

Inkubationszeiten wären langweilig, Arbeitstage monoton, Mittagessen einsam gewesen ohne meine Kollegen aus der Zoologie II. Insbesondere Tom und Conny haben mich vor allem am Anfang meiner Doktorarbeit bei technischen Fragestellungen unterstützt, sodass mir der Einstieg schnell und mit Freude gelungen ist. Für die ganze Schokolade und die aufschlussreichen fachlichen wie nicht fachlichen Plaudereien möchte ich mich bei meinen Zimmermitbewohnern Agustina, Ayse und

Pauline bedanken. Danke auch an alle weiteren Mitglieder der AG Rössler und der Zoologie II, die meine Promotion durch ihre Hilfsbereitschaft und Freundlichkeit bereichert haben. Danke an Jan, Brillo, Strubi, Claudi, Tina, Franzi, Anne, Maren, Mira. Danke an die AG Spaethe inklusive Johannes, Frank und Leo und an die AG Scheiner bestehend aus Ricarda und Thammi, sowie an die gesamte AG Roces und an Frau Linke. Spezieller Dank auch an Dirk, unserem Imkermeister, der mich zuverlässig mit den weltweit zahmsten Honigbienen versorgt hat.

Dank auch an die Graduate School of Life Sciences der Universität Würzburg für die finanzielle Unterstützung.

Während meiner Promotion hatte ich die Möglichkeit, Rebecca und Christoph als Bachelorstudenten zu betreuen und ich möchte mich bei Ihnen für ihre engagierte Hilfe bei meiner Doktorarbeit bedanken.

Besonderen Dank an meine Familie, an Birgit, Manfred und Hermann, die mir den Weg zur Doktorarbeit bereitet haben. Danke an meine Freundin Caroline für die Unterstützung außerhalb des Labors und fürs intensive Korrekturlesen.