Neurophysiological events induced by octopamine and serotonin in the honeybee brain

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Zusammenfassung

Zelluläre Physiologie des olfaktorischen Lernens der Honigbiene (*Apis mellifera*)

Der erste Teil der vorliegenden Arbeit (Kapitel 2) besteht aus einem *Review*-Artikel, der einen Gesamtüberblick über die zelluläre Physiologie des olfaktorischen Lernens der Honigbiene bietet (Himmelreich und Grünewald 2012). In diesem Teil der Arbeit werden die aus Verhaltensexperimenten sowie elektro-physiologischer Studien am Bienengehirn gewonnenen Erkenntnisse über grundlegende zelluläre Strukturen während des Lernvorgangs, wie die Rezeptoraktivierungen und die intrazellulären Signalkaskaden vorgestellt und vor dem Hintergrund der Forschungsergebnisse verschiedener Autoren diskutiert. Bei den meisten Studien zu diesem Themenbereich handelt es sich um Untersuchungen, die bei der klassischen Konditionierung des Proboscisreflexes (Rüsselreflexes) der Honigbiene durchgeführt wurden. Bei der klassischen Konditionierung wird ein unkonditionierter Stimulus (US) in Form eines Zuckertropfens der Proboscis und/oder den Antennen präsentiert und dieser mit einem Duft gepaart. Bei erfolgreicher Konditionierung wird der Duft zum konditionierten Stimulus (CS). Die konditionierte Antwort (CR) ist das reflexartige Ausstrecken der Proboscis. Der Duft-Signalweg wird über olfaktorische Rezeptorneurone (ORN) der Antenne an die Anntenal-Loben vermittelt, die erste Neuropilstruktur, in der Lern- und Gedächtnisprozesse ablaufen. Im Antennal-Lobus bilden die ORN Synapsen mit lokalen Interneuronen und Projektionsneuronen, und setzen an diesen den exzitatorischen Transmitter des Bienengehirns, Acetylcholin, frei. Die Projektionsneurone der Antennal-Loben leiten über antenno-cerebrale Trakte die Duftinformationen an die Pilzkörper weiter, die zweite Neuropilstruktur, in der Lern- und Gedächtnisprozesse ablaufen. Im Pilzkörper befinden sich die intrinsischen Pilzkörperneurone, die Kenyon Zellen. Der Belohnungssignalweg wird durch das ventral-ungepaarte mediale Neuron 1 des maxillaren Neuromers (VUMmx1) vermittelt, dessen Somata im Suboesophagial-ganglion liegt. Das oktopaminerge VUMmx1 bildet Synapsen in den Antennal-Loben sowie in den Pilzkörpern aus. Dies kann dazu führen, dass es in beiden Neuropilstrukuren zu einer Konvergenz beider Stimuli des konditionierten Stimulus und des unkonditionierten Stimulus auf ein einzelnes Neuron kommt.

Der Prozessablauf auf zellulärer Ebene und die Detektion der konvergenten Stimuli ist noch nicht gut untersucht. Im ersten Teil der vorliegenden Arbeit (Kapitel 2) wird hierzu ein Membranmodell vorgestellt, welches zelluläre Mechanismen der Konvergenz in Antennal-Loben Neuronen sowie Kenyon Zellen vorstellt: 1. Der Signalweg des Dufts aktiviert die nACh-Rezeptoren (nAChR) an Antennal-Loben Zellen oder Kenyon Zellen. Dadurch wird ein Einstrom von positiven Ionen (Ca^{2+} und Na^{+}) und damit ein Strom induziert. 2. Gleichzeitig werden durch den Belohnungssignalweg Oktopaminrezeptoren (G-Protein gekoppelte Rezeptoren) aktiviert. In Kenyon Zellen sowie in Antennal-Loben-Neuronen befinden sich α- oder β-adrenerg-ähnliche Oktopaminrezeptoren. Ein α-adrenerg-ähnlicher Oktopaminrezeptor (z.B. der identifizierte und pharmakologisch beschriebene AmOA1 Rezeptor) führt zu einer Aktivierung der Phospholipase C (PLC) und dadurch zu einer Freisetzung von Inosit-1,4,5-triphosphat (IP_3) induziertem Ca^{2+}. Ein möglicher β-adrenerg-ähnlicher Oktopaminrezeptor (noch nicht molekular identifizierter, jedoch durch verschiedene Studien angenommener Rezeptor) führt zu einem intrazellulären zyklischen Adenosinmonophosphat/Proteinkinase A (cAMP/PKA) Signalweg, der durch eine Aktivierung der Adenylylcyclase induziert wird. 3. Bei der Konvergenz des konditionierten Stimulus (CS) und des unkonditionierten Stimulus (US) auf ein Neuron würde der nAChR und einer der beiden Oktopaminrezeptoren oder auch beide aktiviert. Beide Signalkaskaden der Oktopaminrezeptoren könnten den Acetylcholin-induzierten Strom modellieren. In einem Fall würden bei einem verstärkten intrazellulären Anstieg der Kalziumionen-konzentration, welche durch den Einstrom von Kalziumionen (Ca^{2+}-Influx) durch den nAChR sowie dem Ausstrom von Kalziumionen (Ca^{2+}-Efflux) aus intrazellulären Speichern hervorgerufen wird, Ca^{2+}-abhängige Kinasen aktiviert werden. Diese könnten den nAChR modellieren. Im anderen Fall würde bei der Aktivierung des cAMP/PKA Signalweges die PKA den nAChR phosphorylieren. Beide Kinasen könnten demnach Koinzidenzdetektoren des konditionierten und unkonditionierten Stimulus sein. Das Membranmodell wurde im Verlauf der Untersuchungsergebnisse weiterentwickelt. Serotoninrezeptoren, die ähnliche intrazelluläre Signalwege wie die Oktopaminrezeptoren induzieren, wurden in das Modell eingefügt (siehe Kapitel 3).

Oktopamin und Serotonin als Neuromodulatoren in kultivierten Neuronen aus den Pilzkörpern und den Antennal-Loben der Honigbiene

Im zweiten Teil der vorliegenden Arbeit (Kapitel 3) werden einzelne Hypothesen des vorgestellten Membranmodells überprüft. Dazu wurden zwei Untersuchungsreihen an kultivierten puppalen Primärneuronen der Honigbiene (*Apis mellifera carnica*) durchgeführt. Bei der ersten Untersuchungsreihe wurde die Methode des bildgebenden Verfahren des Ca^{2+}-*Imaging* genutzt. Die zweite Untersuchungsreihe wurde mittels der *Patch-Clamp*-Technik durchgeführt.

Erstmalig konnte mit quantitativen Ca^{2+}-*Imaging*-Experimenten starke Ca^{2+}-Signale in Antennal-Lobus-Neuronen sowie in Kenyon Zellen nach der Applikationen von Oktopamin und Serotonin nachgewiesen werden. Die Applikationsdauer einer Messung von 150 s betrug 400 ms. In dieser Zeit wurde 1 µM Oktopamin oder 1 µM Serotonin appliziert. Die Aufnahmezeit der *Imaging*-Apparatur betrug 150 s, bei einer Aufnahmefrequenz von 10 Hz. Die Ergebnisse lassen auf die Aktivierung von G_q-Protein gekoppelten Rezeptoren schließen, was über die Aktivierung der Phospholipase C (PLC) zu eine Freisetzung des *second messengers* IP_3 und zu einer anschließenden Freisetzung von Kalziumionen aus intrazellulären Speichern führt. Die Messungen zeigen unterschiedliche Ca^{2+}-Signale in Kenyon Zellen und Antennal-Lobus-Neuronen: Kenyon Zellen exprimieren Ca^{2+}-Signale mit ausgeprägtem oszillierenden Charakter. Antennal-Lobus-Neuronen exprimieren eher stetig ansteigende Ca^{2+} Signale. Durch die Anpassung einer Fitkurve an die Messungen der einzelnen abklingenden Ca^{2+}-Signale innerhalb einer oszillierenden Ca^{2+}-Antwort, wurde die Halbwertszeit der einzelnen abklingenden Ca^{2+}-Signale berechnet. Die Halbwertszeiten zeigen signifikante Unterschiede der Abklingrate der Ca^{2+}-Signale in Kenyon Zellen nach Applikation von Oktopamin und Serotonin. Einzelne Ca^{2+}-Signale innerhalb einer gesamten Ca^{2+}-Antwort mit oszillierenden Charakter, klingen nach der Applikation von 1 µM Oktopamin schneller ab als nach der Applikation von 1 µM Serotonin. Der Vergleich der Ca^{2+}-Signale von Antennal-Lobus-Neurone mit denen in Kenyon Zellen zeigt ebenfalls signifikante Unterschiede. Antennal-Loben-Neurone zeigen nach der Applikation von Oktopamin deutlich geringere Abklingraten als Kenyon Zellen. In einer weiteren Untersuchungsreihe wurden Oktopaminrezeptor-Antagonisten (100 µM Mianserin und 100 µM Epinastin) verwendet, um Ca^{2+}-Signale in Kenyon Zellen nach der Applikation von Oktopamin

zu unterdrücken. Die Messungen zeigten eine starke Reduzierung der Oktopamin-induzierten Ca^{2+}-Signale nach der Applikation der Antagonisten. Die Messungen zeigen, dass in Kenyon Zellen Oktopaminrezeptor-Antagonisten auch antagonistisch auf Serotonin-induzierte Ca^{2+}-Signale wirken. Dies ist überaus überraschend, da seit Jahren in verschiedenen Studien Mianserin und Epinastin als potentielle Oktopaminrezeptor-Antagonisten verwenden werden (weitere Oktopamin-spezifischen Antagonisten sind nicht bekannt).

Die Ca^{2+}-Imaging-Messungen an Kenyon Zellen zeigen starke Ca^{2+}-Signale nach der Applikation von 100 µM Acetylcholin. Um die Wirkung von Oktopamin/Serotonin auf die Acetylcholin-induzierten Ca^{2+}-Signale zu untersuchen, wurden hintereinander 1 µM Oktopamin/Serotonin und 100 µM Acetylcholin appliziert. Die Messungen zeigen, dass die Acetylcholin-induzierten Ca^{2+}-Signale nach der Applikation von Oktopamin und Serotonin signifikant schwächer wurden. In einer weiteren Untersuchungsreihe wurden Messungen an Kenyon Zellen und Antennal-Lobus-Neurone im *Whole*-Zell-Modus mit der *Patch-Clamp*-Technik durchgeführt. Die *Patch-Clamp*-Messungen bestätigen die Ergebnisse der Untersuchungen mittels des Ca^{2+}-*Imaging*-Verfahrens. Bei den *Patch-Clamp*-Messungen wurden die Zellen bei -70 mV Haltepotential „geklemmt“. Zunächst wurde nach der Applikation von 100 µM Acetylcholin (400 ms) ein starker, in die Zelle gerichteter Strom gemessen. Anschließend wurden (1 µM) Oktopamin oder (1 µM) Serotonin über die Badperfusionslösung für 135 s appliziert und der Acetylcholin-induzierte Strom erneut gemessen. Die dritte Messung wurde nach weiteren 135 s durchgeführt, nachdem der Transmitter aus dem Bad herausgespült wurde. Die Maxima der Acetylcholin-induzierten Ströme nehmen nach der Applikation von Oktopamin relativ zum Maxima der ersten Strommessung signifikant ab, wobei diese Abnahme in Kenyon Zellen stärker als in Antennal-Lobus-Neuronen erfolgt. Nach Applikation von 1 µM Serotonin nimmt der Acetylcholin-induzierte Strom in Antennal-Lobus-Neuronen weiter ab, wobei dieser Effekt stärker ist als die erst gennannte Abnahme infolge der Applikation von Oktopamin. In einem nächsten Schritt wurden die gleichen Messungen durchgeführt nachdem 10 µM der membrangängigen Substanz Forskolin appliziert wurde. Forskolin kann eine Adenylylcyclase aktivieren. Die Ergebnisse der Messungen, deuten auf die Beteiligung einer Adenylylcyclasenaktivierung hin, die durch einen möglichen β-adrenerg-ähnlichen Oktopamin-Rezeptor induziert werden

könnte. Eine gleichzeitige Aktivierung der Rezeptoren durch den konditionierten Stimulus-Signalweg und den Belohnungssignalweg führt zu einer Modulation des Acetylcholin-induzierten Ca^{2+}-Signals bzw. des in die Zelle gerichteten Stroms. Diese Ergebnisse bestätigen die Hypothesen aus dem 1. Teil der Arbeit (*Review*-Artikel, Kapitel 2 der vorliegenden Arbeit) und lassen auf eine intrazelluläre Signalkaskade schließen, die in einer möglichen nACh-Rezeptor Modulation mündet und dadurch zu einer Änderung der Rezeptorleitfähigkeit führt.

In vitro CREB Stimulierung in Kenyon Zellen der Honigbiene

Dem dritten Teil der vorliegenden Arbeit (Kapitel 4) liegt die Hypothese zu Grunde, dass durch die Aktivierung von Oktopamin- oder Serotoninrezeptoren, die ein cAMP/PKA Signalweg über eine Adenylylcyclase veranlassen, eine Phosphorylierung des Transkriptionsfaktors CREB (*cAMP-response element-binding protein*) auslösen. Dabei würde in Kenyon Zellen die Aktivierung eines β-adrenerg-ähnlichen Oktopaminrezeptor zur möglichen Phosphorylierung von CREB führen. Bei einer Aktivierung der Serotoninrezeptoren würde es zu einer möglichen Phosphorylierung oder zur Dephosphorylierung von CREB führen, abhängig welcher der Serotoninrezeptoren aktiviert wird. Zur Untersuchung der Stimulierung der Phosphorylierung von CREB wurden Messungen an Kenyon Zellen mittels immunzytologischer Methode durchgeführt. CREB wurde dabei für 0.5 min, 10 min und 60 min stimuliert. Zur Kontrolle wurde eine Gruppe nur mit Ringer-Lösung versehen und der gleichen Zeitreihemessung unterzogen. Einer weiteren Kontrollgruppe wurde die Fixierlösung und die Stimulanz gleichzeitig gegeben („0 min“ Kontrolle). Bis auf die Gruppe, welche für 0.5 min mit Serotonin stimuliert wurde, zeigt keine andere Gruppe signifikante Unterschiede des phosphorylierten CREB-Levels relativ zur Kontrollgruppe des gleichen Zeitintervalls. Die Gruppe, welche für 0.5 min mit Serotonin stimuliert wurde, zeigt eine signifikante Erniedrigung des phosphorylierten-CREB-Levels. Hier könnte ein Serotoninrezeptor aktiv sein, wie der 5HT1A Rezeptor, der einen cAMP/PKA Signalweg inhibiert und damit die Phosphorylierung von CREB herunterreguliert.

CHAPTER 1

1 Introduction

1.1 Learning and memory formation

The survival of animals in nature depends upon their adapting to the environment in the most balanced way in terms of energy consumption. Learning constitutes one adaptation which enables the animal to deal with recurring environmental events. It allows the animal to adapt behaviorally to changing situations, including activities like locomotion, foraging, mating, grooming, or communication. Memory formation initially causes an expenditure of energy, but over the time, it entails the ability to form a retrievable mechanism which, in the end, saves energy with its prestructured neuronal compartments and efficient connectivities. Hence, learning can be defined as consistent process that results in adaptive change in behavior. This effects the neuronal development and the nervous system and leads to memory formation. Learning is the capacity to store information which is retrievable and therefore of great value for the animal (Squire 1987).

Successful learning requires a high accuracy in memory processing, which in turn requires the reliable computation of a given environmental input, correctly correlating it to a response. This correct input-output connectivity underlies stimuli-dependent changes in the brain. Disturbances of this balanced system could be caused by neuronal diseases and injuries or neuro-active substances (see Chapter 1.5 General discussion: *Exposition to neuro-active substances during foraging*).

For decades, the learning-induced changes have been considered to result from synaptic plasticity mechanisms (Hebb 1949). The striking work with the sea slug *Aplysia californica* was the key in pinpointing learning-induced morphological changes as proposed by Hebb in an intact nervous system for the first time (for a review, see Kandel 2012). The understanding of the relatively simple neuronal circuitry of the gill- and siphon-withdrawal reflex facilitates the further analysis of the cellular and molecular mechanisms underlying nonassociative and associative learning. Learning-induced changes are the results of gene expression/protein synthesis (Kandel 2001) and are ending up in synaptic strengthening or weakening

(Squire and Kandel 1999, Mayford et al. 2012). First pioneering studies in synaptic plasticity demonstrated that the initial formation of a memory trace in rats depends on the activation of NMDA receptors (Morris et al. 1986). Studies of intracellular pathways which are induced during learning revealed that the intracellular cAMP/PKA pathway, which ends in the phosphorylation or dephosphorylation of the transcription factor, the cAMP response binding protein (CREB), is activated in Aplysia (CREB; Brunelli et al. 1976, Yin et al. 1995, for a review, see Kandel 2012).

To gain a deeper understanding of learning and memory and the underlying neuronal changes, neuroscientists need a suitable model system and learning paradigms. But what constitutes a successful model system and successful learning paradigms in neuroscience?

One challenge in studying learning is that it can still only be measured through its related behavioral response, even though it is already possible to detect neuronal modifications. Because a behavioral response is determined by the interaction of component processes, such as motivation, attention, and perception, the study of learning is not easy to control. Therefore, highly controlled experiments are needed to reliably study learning and memory formation. For years, neuroscientists have focused on classical conditioning, an associative form of learning, in their studies of learning and memory formation. Classical conditioning is one method in neuroscience which allows researchers to transfer the study of learning and memory into the laboratory, thereby making the studies highly controlled and reproducible. In his pioneering work in the beginning of the 20th century, the Russian physiologist Ivan Pavlov discovered the classical conditioning learning paradigm in vertebrates (Pavlov 1927). Many insects, including the honeybee, can be classically conditioned. This paradigm enables the systematic study of learning and memory and allows the manipulation of neural processes while simultaneously recording physiological and behavioral responses. Therefore, many pioneering works have used classical conditioning to discover the underlying neuronal networks and cellular mechanisms which lead to learning and memory formation in flies and honeybees (for reviews, see Giurfa and Sandoz 2012, Perry and Barron 2012, Menzel 2012, Busto et al. 2012).

1.2 The honeybee as a model system for studying learning and memory formation

The honeybee´s simpler form of neuronal structure compared to that of higher-order animals facilitates the study of learning and memory during olfactory conditioning; for example, single memory traces and neuronal changes can be more easily ascribed to particular behavioral responses. Hence, the honeybee, which has a small brain containing 950,000 neurons (1mm^3) yet still demonstrates rich behavior, serves as a good model (Witthöft 1967).

Figure 1. Restrained bees in the laboratory. Bees sitting in small plastic tubes waiting for the rewarding sugar drop. The antennae and the proboscis are free during olfactory conditioning (Image by Uwe Dettmer, 2009).

During olfactory conditioning, the restrained bee (see Figure 1) learns to associate a reward stimulus in the form of a sucrose drop presented to the antennae and to the proboscis and an odorant presented to the antennae (unconditioned stimulus, US). After the bee has been successfully conditioned, the odorant becomes the conditioned stimulus (CS) and the conditioned response (CR) is the reflexive extension of the proboscis (Bittermann et al. 1983). Color stimuli can also be used as US during conditioning, as was done in the first classical conditioning experiments (Kuwabara 1957).

1.3 The honeybee brain areas involved in olfactory learning

During olfactory learning and memory formation, three main neuropils are involved in the honeybee brain (see Figure 2): the antennal lobe, the mushroom body, and the subesophageal ganglion.

The antennal lobe, which is the first order neuropil and an analog to the vertebrate olfactory bulb (Hildebrand and Shepherd 1997, Kay and Stopfer 2006), receives olfactory input from the olfactory receptor neurons (ORN) of the antennae. The projection neurons of the antennal lobe convey the processed information regarding the odor as spatiotemporal response patterns (Sachse and Galizia 2002, Krofczik et al. 2009) to the second order neuropil, the mushroom bodies (i.e., the corpora pedunculata; Mobbs 1982, Starusfeld 2002). The connection between the antennal lobe and mushroom body is unique in hymenoptera. It is a dual olfactory pathway with a medial antennal lobe protocerebral tract (m-APT) and a lateral antennal lobe protocerebral tract (l-APT) which are both comprised of projection neuron axons which connect the antennal lobes with the mushroom bodies (Abel et al. 2001; Kirschner et al. 2006; Zube et al. 2008; Galizia and Rössler 2010). Simultaneous multi-unit recording from both tracts reveals different response profiles, leading to the assumption that odors are processed in parallel: the l-APT projection neurons had broad response profiles suggesting generalized coding properties, whereas the responses of m-APT projection neurons were comparatively weaker and less frequent, indicating higher odor specificity (Brill et al. 2013). About 170,000 small diameter Kenyon cells, the mushroom body intrinsic neurons with their dendritic arborizations comprise the main postsynaptic elements of the mushroom body calyces (Kenyon 1896, Witthöft 1967, Mobbs 1982). Kenyon cells receive multisensory input and convey this input to mushroom body extrinsic neurons, mainly in the alpha- and beta-lobes, providing the main mushroom body output to other brain regions, such as the lateral protocerebrum or the contralateral brain hemisphere (Mobbs 1982, Gronenberg 1987, Rybak and Menzel 1993, Straussfeld 2002). Among the extrinsic neurons there are different subgroups of neurons, such as the GABA-immunoreactive feedback neurons, also called protocerebral-calycal tract neurons (Mobbs 1982, Bicker et al. 1985, Grünewald 1999b), which provide inhibitory feedback to the intrinsic Kenyon cells residing in the mushroom body calyces and also connect the dorsal and median alpha-lobe, the beta-lobe, as well as the pedunculus with ipsilateral calycal subcompartments (Grünewald 1999a). The

individually identified extrinsic neuron PE1 changes its responses due to associated or non-associated odors (Mauelshagen 1993), which suggests that learning-related plasticity also occurs on the extrinsic neuron level.

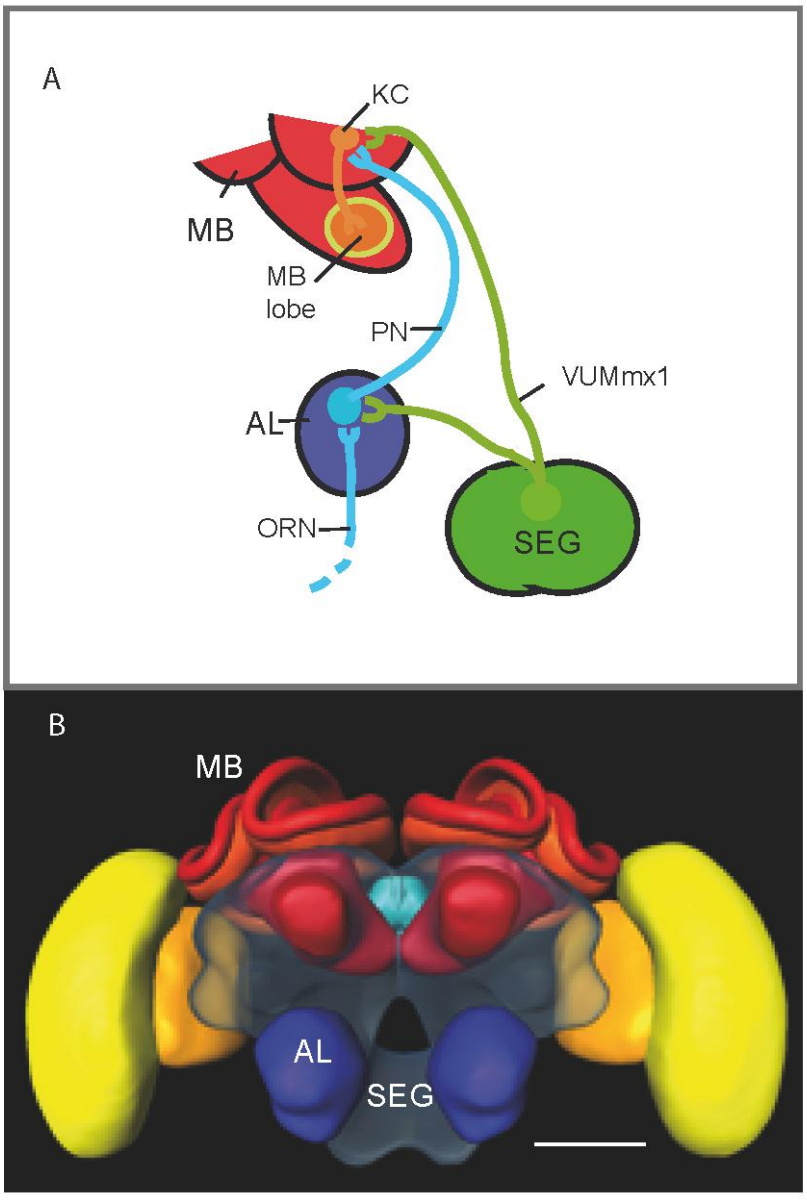

Figure 2 | The honeybee neuropils, the connections housing the input during appetitive olfactory learning, and the honeybee standard brain. *A* Schematic diagram of the three main neuropils involved in reward learning during the conditioning. For simplicity, the diagram depicts only one hemisphere and the input during appetitive olfactory learning, such as the main connections between the

subesophageal ganglion (SEG), antennal lobe (AL) and mushroom body (MB). The sucrose reward is processed in the subesophageal ganglion (in green) and via the ventral unpaired median neuron 1 of the maxillary neuromere (VUMmx1, light green) the reward signal is sent to higher-order neuropils - the antennal lobe (blue) and the mushroom body (red). The olfactory conditioned stimulus (CS) is conveyed via olfactory receptor neurons of the antennae (ORN, light blue) to the antennal lobe. Here, the CS and reward signal converge on the same neurons for the first time. Projection neurons (PNs, light blue) leave the antennal lobe via antenno-cerebral tracts to the mushroom body input region. Projection neurons synapse onto Kenyon cells (KCs, orange), the mushroom body intrinsic neurons, and may converge again with the reward signal on the same neuron. The axons of Kenyon cells descend via the mushroom body peduncle and converge on the mushroom body extrinsic neurons (not shown) within the peduncle and the mushroom body lobes (orange). *B* shows a surface reconstruction of the honeybee standard brain. All the neuropils are shown in different colors. The mushroom body and the antennal lobe are colored similarly as in *A*. The subesophageal ganglion is shown in light grey. Scale bar: 300 µM. (Figure 2*B* has been modified with permission from Rybak et al. 2010.)

1.4 Main findings and their significance

Cellular physiology of olfactory learning in the honeybee

The first part of the current work is a review article which provides an overview on the cellular physiology of olfactory learning in the honeybee (Himmelreich and Grünewald 2012). This part of the work presents and discusses the findings from various studies on the pivotal cellular structures, processes, and networks associated with olfactory learning, such as receptor activation and intracellular pathways. In most studies, the findings were based on pioneering experiments involving the olfactory conditioning of the honeybee´s proboscis extension reflexes (Bittermann et al. 1983, Menzel 1990; Menzel and Müller 1996, Mauelshagen 1993; Hammer and Menzel 1998; Szyszka et al. 2008), therefore providing insight into the cellular and neuronal networks related to learning and memory events. The cellular processes involved in olfactory conditioning and the detection of the possible stimuli convergence are not yet well understood. Besides reviewing the findings from electrophysiological studies, the review article also presents a cellular model of the underlying processes occurring during possible stimuli convergence and the underlying cellular pathways. This model describes a possible convergence of the conditioned stimulus and unconditioned stimulus on the same neuron, such as neurons in the antennal lobe or in the mushroom body, where the neurons of the olfactory and reward pathway could

convey their information to same neurons. Still less is known about the process operation and detection during the convergence of the stimuli. In Chapter 2, the model of possible cellular mechanisms underlying the convergence of the olfactory and reward pathway in antennal lobe neurons and Kenyon cells is presented:

1. The olfactory pathway activates the nicotinic acetylcholine receptors within the antennal lobes and mushroom bodies. Therefore, an activating inward current of positive ions (calcium and sodium) is induced.
2. Coincidently with the activation of the nicotinic acetylcholine receptors, octopamine receptors (G-protein coupled receptors) are activated by the reward pathway. In Kenyon cells as well as in antennal lobe neurons, the α- and β-adrenergic-like octopamine receptors are expressed. The α-adrenergic-like octopamine receptor, such as the molecularly and functionally described AmOA1, is coupled to the G_q-protein and induces an activation of the phospholipase C (PLC) and therefore a release of inosit-1,4,5-triphosphate (IP_3). The IP_3 in turn induces the Ca^{2+} efflux out of the endoplasmic reticulum. The activation of a possible β-adrenergic-like octopamine receptor (there is still no molecularly described receptor, but one has been assumed by various studies) leads to an intracellular cyclic adenosinmonophosphase/protein kinase A (cAMP/PKA) pathway mediated by the activation of the adenylyl cyclase.
3. The model assumes that during the convergence of the conditioned stimulus and unconditioned stimulus on one neuron, both receptor types, namely the nicotinic acetylcholine receptor together with one or both octopamine receptors, are activated.

The acetylcholine-induced current could be modulated by the activation of both signaling pathways, either by the α- or β-adrenergic-like octopamine receptor activation. When the α-adrenergic-like octopamine receptor is activated, the intracellular Ca^{2+} increases due to the Ca^{2+} efflux out of internal stores and due to the Ca^{2+} influx through the nicotinic acetylcholine receptor into the cell lumen. Therefore, unknown Ca^{2+}-dependent kinases could be activated. If the β-adrenergic-like octopamine receptor were activated, the cAMP/PKA pathway would be activated and the PKA itself could be the agent to phosphorylate the nicotinic acetylcholine

receptor. Both kinases would serve as possible coincidence detectors for the concurrence conditioned stimulus and unconditioned stimulus.

During the presented work, we have improved the model of the underlying cellular physiology of learning by adjusting the model to reflect our new findings, for example, by adding the serotonin receptor to the model (Chapter 3).

Octopamine and serotonin as neuromodulators in the cultured neurons of the honeybee mushroom body and antennal lobe

In Chapter 3 the hypotheses of the cellular-physiology model are examined. For this purpose, two investigations were conducted with a primary cell culture of honeybee pupae. In the first investigation, we used the Ca^{2+}-imaging approach and in the second, the patch-clamp technique.

For the first time in quantitative Ca^{2+}-imaging experiments, we show strong Ca^{2+} signals in antennal lobe neurons and in Kenyon cells after the application of 1-µM octopamine and 1-µM serotonin (400-ms application with a multibarrel micropipette). The Ca^{2+} signals were recorded for 150 s at a sampling rate of 10 Hz. The findings suggest an activation of the G_q-coupled octopamine receptor, which leads to a release of IP_3, mediated by the activation of the phospholipase C and ending in a Ca^{2+} efflux out of internal stores. The results show that there are different Ca^{2+} signals in Kenyon cells than in antennal lobe neurons: Kenyon cells express Ca^{2+} signals with a distinct oscillating character, whereas antennal lobe neurons express Ca^{2+} transients that are slowly increasing.

By fitting the collected data with a curve, the half-life of the Ca^{2+} decay in each Ca^{2+} response was calculated. The half-life values show significant differences in the Ca^{2+} decay in Kenyon cells after the application of 1-µM octopamine and the application of 1-µM serotonin. The Ca^{2+} decays in the Kenyon cells after the 1-µM octopamine application are faster than after the 1-µM serotonin application. The comparison of Ca^{2+} signals in the antennal lobe neurons and Kenyon cells also shows significant differences. After the application of 1-µM octopamine, the antennal lobe neurons have lower decay times than the decay times in the Kenyon cells. These results suggest that the octopamine receptors and serotonin receptors may have different

expression levels. They could also induce different intracellular pathways in Kenyon cells than in antennal lobe neurons.

We also tested the octopamine receptor antagonists mianserin and epinastin, which were both applied in a concentration of 100 µM. These antagonists inhibited the octopamine-induced Ca^{2+} signals. Additionally, these antagonists also had an inhibiting effect on the serotonin-induced Ca^{2+} signals in Kenyon cells. These findings are significant because various behavioral studies have used epinastin and mianserin as potential antagonists of the octopamine receptor without considering any possible effect on the serotonin receptor.

In the Ca^{2+} imaging approach, we find strong Ca^{2+} signals after applying 100-µM acetylcholine. In our investigation of the octopamine/serotonin effects on the acetylcholine-induced Ca^{2+} signals in Kenyon cells, we successively applied acetylcholine as the prepulse, then octopamine and then again acetylcholine. The acetylcholine-induced Ca^{2+} signals decreased after the octopamine or serotonin were applied.

In further measurements, we used the patch-clamp technique in whole-cell mode to analyze the octopamine/serotonin influences on the acetylcholine-induced currents in Kenyon cells and antennal lobe neurons. The results of these measurements support the findings from the Ca^{2+} imaging recordings. The cells were clamped by -70 mV, the resting potential of the neurons. Three consecutive acetylcholine applications with an interstimulus interval of 135 s were performed. Between the first and second acetylcholine applications, either 1-µM octopamine or serotonin was applied via bath perfusion. Between the second and third acetylcholine applications, the octopamine or serotonin was washed out. After the octopamine application, the peak currents decreased significantly relative to the peak current after the first acetylcholine application in Kenyon cells and in antennal lobe neurons, with the currents in Kenyon cells decreasing more strongly than the currents in antennal lobe neurons. After the 1-µM serotonin application, the acetylcholine-induced current also decreased in the antennal lobe neurons; however, this decrease was greater than the decrease measured after the octopamine application. In an additional experiment, the adenylyl cyclase activator forskolin (10 µM) was applied using the same procedure as with octopamine or serotonin. The findings suggest the activation of an adenylyl cyclase

which leads to a cAMP/PKA pathway, possibly to a similar pathway as the one induced by the β-adrenergic-like octopamine or by similar serotonin receptors. This intracellular pathway leads to a modulation of the nicotinic acetylcholine receptor and therefore to a decrease in the acetylcholine-induced current. These findings support the hypothesis proposed in the first part of the presented work (Chapter 2, Himmelreich and Grünewald 2012) and indicate that the intracellular pathways lead to a nicotinic acetylcholine receptor modulation, thereby changing the receptor conductance.

In vitro CREB stimulation of honeybee Kenyon cells

In the third part of the presented work (Chapter 4), the following hypothesis underlies our study on phosphorylated-CREB stimulation: By activating specific octopamine or serotonin receptors, adenylyl cyclases are induced to activate a cAMP/PKA pathway which ends in the phosphorylation of the transcription factor CREB. Therefore, in Kenyon cells, the activation of a β-adrenergic-like octopamine receptor leads to the phosphorylation of CREB. The activation of serotonin receptors, such as the 5HT1A receptor, leads to an inhibition of the cAMP/PKA pathway (Thamm et al. 2010) and therefore to a dephosphorylation of CREB. The activation of another serotonin receptor, the 5HT7, also induces the cAMP/PKA pathway, hence leading to the phosphorylation of CREB.

To investigate the phosphorylation of CREB after octopamine and serotonin stimulations in Kenyon cells, we used the immunocytochemical approach to detect phosophorylated CREB. The CREB was stimulated with the transmitters for 0.5 min, 10 min, and 60 min. As a control, we had groups which were only stimulated with saline solution and some groups which received the transmitters and the fixation solution simultaneously. The experimental group which was stimulated with 1-µM serotonin for 0.5 min showed a significant decrease in phosphorylated CREB level. No other group showed remarkable differences in phosphorylated CREB levels compared to the respective control group of the same time interval. This leads to the assumption that the 5HT1A receptor is activated, leading to an inhibition of a cAMP/PKA pathway and therefore to a decrease in phosphorylated CREB during the 0.5-min stimulation.

1.5 General discussion

Cellular physiology of learning-related events

The findings indicate a modulatory interaction between the acetylcholine receptor and the octopamine receptor as well as between the acetylcholine receptor and the serotonin receptor, despite the still unclear role of serotonin in learning. Further, the findings may suggest how the signals of the conditioned stimulus and unconditioned stimulus converge and are processed on the cellular level. These modulatory interactions may be the first step in learning-related events.

Considering the findings of strong octopamine-induced Ca^{2+} signals and no remarkable differences in the octopamine-induced phosphorylated CREB levels in Kenyon cells, one could assume that the octopamine receptors receiving input from the reward neuron, the VUMmx1 neuron, are more likely coupled to Ca^{2+}-regulated pathways than to the cAMP/PKA pathway.

The finding of no significant differences in phosphorylated CREB levels after octopamine applications in Kenyon cells is puzzling, because mushroom body neurons are assumed to be the place of long-term memory formation and the phosphorylation of CREB, followed by gene transcription and de novo protein synthesis, are the underlying long-term-memory-formation events (see reviews for honeybees: Menzel 2012, Giurfa 2007; see reviews for *Drosophila* Keene and Waddell 2007; Davis 2011). The most obvious explanation of these results would be that in a reduced *in vitro* preparation of primary cell culture, the phosphorylation of CREB cannot be induced by simply activating the octopamine receptor. Another explanation is the possibility of incorrect timing for the stimulations. However, decreased phosphorylated CREB was observed after the 0.5-min serotonin stimulation, indicating that it was possible to observe changes in phosphorylated CREB levels in our preparation. Future studies could vary the time intervals of the stimulations and also stimulate the phosphorylation of CREB by simultaneously activating the following two receptors which are discussed throughout the present work: the acetylcholine receptor for the olfactory pathway and the octopamine receptor for the reward pathway. Possibly then the phosphorylation of the CREB levels would increase as a result of the synergistically induced intracellular pathways (the same pathways as assumed in Chapter 3) in the modulatory interactions by the

simultaneous activation of the nicotinic acetylcholine receptor and the octopamine receptor.

Synthesis of findings - cellular model of integration of the olfactory and reward pathway during olfactory learning

The model describes the convergence of the olfactory pathway and reward pathway on antennal lobe neurons or mushroom body Kenyon cells and depicts the receptor activations, intracellular mechanisms, and modulatory changes that may occur during learning-related events (Figure 3). The possible integration between the olfactory pathway and the reward pathway, is described. The model also includes the findings from the Chapter 4 (*In vitro* CREB stimulations of honeybee Kenyon cells) and therefore incorporates possible serotonin receptor activation, which inhibits the phosphorylation of CREB.

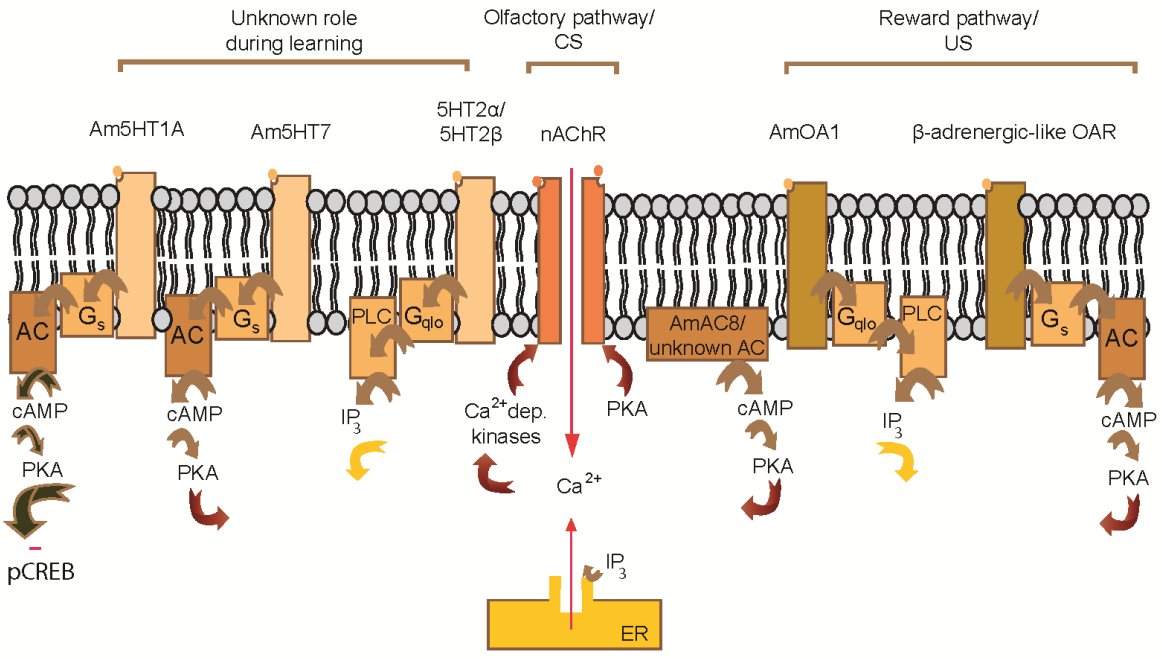

Figure 3 | Cellular integration model of the olfactory and reward pathway during olfactory learning. The schematic diagram depicts the cellular physiology in

Kenyon cells and antennal lobe neurons where the unconditioned stimulus (US) and conditioned stimulus (CS) pathways could converge. The olfactory pathway activates the nicotinic acetylcholine receptor, leading to a strong Ca^{2+} and sodium influx and therefore depolarizes the postsynaptic membrane in antennal lobe neurons and Kenyon cells. The reward signal could activate either the octopamine (AmOA1) receptor, which induces a Ca^{2+} efflux out of the endoplasmic reticulum (ER) by mediating intracellular inositol triphosphate (IP_3) release. Or the reward signal could activate an identified but not yet characterized β-adrenergic-like octopamine receptor, which mediates a cAMP/PKA signal through a receptor positively coupled to an adenylyl cyclase (indicated as AC). Both pathways could have modulatory effects on ACh-induced inward currents by activating a Ca^{2+}-dependent kinase or a protein kinase A (PKA) to modulate the nicotinic acetylcholine receptor and lead to modulatory conductance changes (for further explanations see Chapter 3). These kinases may therefore act as coincidence detectors of the convergence of the CS and US during conditioning. The role of serotonin receptors during learning are still unknown. But the indicated serotonin receptors (5HT2α/5HT2β and Am5HT7) may induce intracellular pathways similar to those of the two OA receptors. By stimulating the serotonin receptor, possibly the Am5HT1A receptor, the cAMP response element-binding protein (CREB) decreases (for further explanations see Chapter 4). (Light brown arrows indicate the activity-dependency and yellow arrows stand for the IP_3 signaling pathway. Red arrows indicate the possible directly or indirectly modulatory effect on the nicotinic acetylcholine receptor. Dark brown arrows indicate a negative influence.)

Exposition to neuro-active substances during foraging

The honeybee has a pivotal role in agriculture as one of the main pollinators in Central Europe and in other continents, like North America and Asia. Honeybees pollinate various cultured plants, such as canola, sunflower, and various fruits, and in doing so, they ensure a significant increase in the crop yield. Therefore, honeybee health is of great economical value, and the economy is interested in keeping the negative influences of agricultural insecticides on the honeybee as low as possible. Besides all the synthetically produced insecticides, there is also a common natural botanical repellent, the tobacco plant *Nicotiana tabacum*, with nicotine acting on the insect acetylcholine receptor. The commonly used synthetic neonicotinoid insecticides have structures chemically similar to nicotine and are produced to control agriculturally important crop pests. They are therefore designed to prevent, destroy, repel, or kill insects. The selectivity of neonicotinoid compounds for insect species has been attributed to their binding on the nicotinic acetylcholine receptors, in which

negatively charged nitro- or cyano-groups of neonicotinoid compounds interact with the cationic subsite within the insect nicotinic acetylcholine receptors (for review see Thany 2010). The insecticides mainly used are neonicotinoids, such as imidacloprid, thiacloprid, thiamethoxam, and clothianidin (for a review, see Decourtye and Devillers 2010), whose influences have been investigated in the last years because their use in agriculture has been implicated in insect pollinator population decline.

Electrophysiological studies have shown that imidacloprid acts as a partial agonist of the nicotinic acetylcholine receptor of honeybee Kenyon cells (Déglise et al. 2002) and clothianidin as a *superagonist* on the insect nicotinic acetylcholine receptor (Brown et al 2006). Using recordings from mushroom body Kenyon cells in acutely isolated honeybee brain, it was shown that the neonicotinoids imidacloprid and clothianidin cause a depolarization block on neuronal firing and inhibit nicotinic responses (Palmer et al. 2013). Behavioral studies also showed that even under sublethal concentrations, the neonicotinoids have strong effects on flight duration and foraging (Henry et al. 2012; Schneider et al. 2012). Closer study of the flight behavior showed effects on navigational skills and choosing the correct path. Even with respect to colony traits, neonicotinoids have effects on proper brood development (Fischer pers. comm.).

Other insecticides such as the two formamidines *demethylchlordimeform* and *amitraz,* and the essential oil *thymol* are used for the treatment of varroosis (*Varroa destructor*, the parasitic mite of the honeybee). All of these substances act specifically on the octopamine receptor by being highly toxic for the mite but only slightly toxic for bees (reviewed by Blenau et al. 2011).

Regarding the findings of the present work that the acetylcholine receptor and the octopamine receptor may be essential for learning-related events in the honeybee brain, one could image the possible negative influences of insecticides on the balanced system of receptor interaction during learning-related events. Any disturbances involving the activation or inhibition of the nicotinic acetylcholine receptor or the octopamine receptor may have devastating consequences on learning and memory formation, for example, during the orientation and foraging of the honeybee. It is essential that studies be done regarding the influences of the previously mentioned insecticides, including the influences on neuronal mechanisms

as well as the influences on the individual and colony traits. The findings of these studies may give insight into the possible causes of the current declines in honeybee and other wild bee populations.

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CHAPTER 2[1]

2 Cellular physiology of olfactory learning in the honeybee brain

2.1 Abstract

The honeybee (*Apis mellifera*) is a model organism for the study of learning and memory formation and its underlying cellular mechanisms. The neuronal and molecular bases of olfactory associative learning have been intensively studied using the proboscis-extension-reflex (PER) conditioning. The neuronal pathway of the associative olfactory learning includes two main neuropils: the antennal lobes (AL) and the mushroom bodies (MB). Here, the excitatory olfactory and octopaminergic reward pathway converge together onto the AL neurons and MB intrinsic Kenyon cells (KCs). For learning-related neural plasticity to occur the coincidence between the conditioned stimulus (CS) and the reward has to be reliably detected. Therefore, this review focusses on (1) the excitatory ionotropic nicotinic-acetylcholine receptor (nAChR) and (2) the metabotropic octopamine receptor (OAR) which are located on the cell membrane in AL neurons as well as in KCs. For plasticity dependent cellular mechanisms we discuss the role of inhibition provided by GABAergic local interneurons in the ALs and feedback neurons in the MBs, as well as glutamatergic neurons in both neuropils. In our working model we postulate two possible coincidence detector systems which may modulate further incoming olfactory stimuli: (1) An elevated intracellular Ca^{2+} concentration induced by the activation of the nAChR and OAR may result in the activation of a Ca^{2+}-dependent kinase. (2) Activation of a cAMP-dependent PKA may lead to phosphorylation of the nAChR and hence to learning-related intracellular changes.

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Sophie Ziegler-Himmelreich wrote the manuscript and designed and developed all figures and tables. Bernd Grünewald provided intensive suggestions, criticisms and correction on the text as well on the figures.

2.2 Introduction

Learning processes result in memory formation that leaves traces within the central nervous system. Thus, environmental changes are detected and translated into changes of the electrical properties of single neurons and the synaptic communication within neural networks. The search for the neural bases of learning and memory includes analyses of physiological processes on a molecular and cellular level.

The honeybee, is a valuable invertebrate model organism for research on cellular mechanisms of learning and memory (for a review, see Menzel 1999) and is used to investigate insecticide effects on cognitive behavior (for reviews, see Belzunces et al. 2012, this issue and Blenau et al. 2011 this issue). Honeybees can be conditioned to extend their probosces (proboscis extension response, PER) in expectation of an odor signaled food reward (appetitive odor learning: Menzel et al. 1974; Bitterman et al. 1983). A single pairing of the unconditioned stimulus (US; sucrose solution), applied to the antenna and the proboscis following an odor stimulus (conditioned stimulus, CS) is sufficient for the bee to learn that the CS predicts the occurrence of the reward. A single conditioning trial induces a memory that decays within several days and is sensitive to amnesic treatments. Multiple training trials may lead to a long lasting protein synthesis-dependent olfactory memory (Menzel et al 1974; Grünbaum et al. 1998; Wüstenberg et al. 1998; Friedrich et al. 2004; for a review, see Müller 2012 this issue).

The physiological bases of this olfactory learning within the honeybee brain are well described (Hammer 1997; for a review, see Hammer et al. 1998); Table I lists studies on physiological studies of olfactory learning related events in various regions of the honeybee brain. During classical conditioning, the CS-induced excitation within neurons of the AL and the MB are modulated. This modulation depends on the correct timing (contiguity) and a positive contingency between the CS and the reward. In the honeybee subesophageal ganglion, the VUMmx1 (ventral unpaired median neuron of the maxillary neuromere) neuron was identified to be crucially involved in the reward-mediating pathway.

This overview focuses on the neurophysiological bases of odor learning in the honeybee. We will discuss functional properties of those electrophysiologically

identified transmitter receptors that mediate fast synaptic transmission (ionotropic receptors for GABA, acetylcholine or glutamate). We hypothesize that synaptic modulation depends on the activation of metabotropic OAR between neurons of the olfactory pathway. These data are implemented into a working model of the cellular mechanisms of olfactory memory formation in the honeybee brain.

Table I. List of physiological studies of olfactory learning related events in various regions of the honeybee brain.

Brain region/ cell type	Method	Key findings	Author/s
SOG: VUMmx1 neuron	*in vivo* intracellular recordings	VUMmx1 neuron mediates the reinforcement in associative learning.	(M. Hammer 1993)
AL: glomeruli; PNs	*in vivo* Ca^{2+}-imaging	AL rearrange neural representation.	(Deisig et al. 2010)
AL: glomeruli, PNs	*in vivo* Ca^{2+}-imaging	Glomerulus specific changes in PN response strength.	(Rath et al. 2011)
AL: glomeruli	*in vivo* Ca^{2+}-imaging	Rewarded odors activate specific glomeruli more than unrewarded ones.	(Faber et al. 1999)
AL: glomeruli	*in vivo* Ca^{2+} imaging	Increasing differences of activation patterns during differential conditioning.	(Fernadez et al. 2009)
AL: uniglomeruli PNs	*in vivo* Ca^{2+}-imaging	PNs are reliable for odor coding, but are not modified by learning.	(Peele et al. 2006)
AL: lateral PNs, multiglomeruli PN	*in vivo* Ca^{2+}-imaging	mPN: less odor-concentration dependence, narrow tuning profiles; lPN: high odor-concentration dependence, broader tuning profile.	(Yamagata et al. 2009)

AL: PNs MB: KCs; boutons	*in vivo* Ca^{2+}-imaging	Sparsening and temporal sharpening of KCs responses to learned odors.	(Szyszka et al. 2005)
MB: lip region	*in vivo* Ca^{2+}-imaging	Rewarded odors activate MB KCs more than unrewarded.	(Faber et al. 2001)
MB: KCs	*in vivo* Ca^{2+}-imaging	Odor-reward pairing leads to prolongation of responses. KCs response to learned odors is different from KCs responses to unlearned odors.	(Szyszka et al. 2008)
MB: ENs	*in vivo* extracellular recordings	„Switching“ and „modulated“ ENs during retention tests.	(Strube-Bloss et al. 2011)
MB: PE1 neuron	*in vivo* extracellular recordings	PE1 neuron decreases activity during olfactory conditioning.	(Okada et al. 2007)
MB: PE1 neuron	*in vivo* intracellular recordings	Identification of PE1 neuron; learning related response patterns.	(Mauelshagen 1993)
MB: PE1 neuron	*in vivo* intracellular recordings	Associative LTP in the PE1 neuron.	(Menzel et al.2005)
MB feedback neurons (A3 cluster)	*in vivo* Ca^{2+}-imaging	Odor-concentration dependent activity; CS+ activity decrease slower during extinction tests as CS-.	(Haehnel et al.2010)
MB feedback neurons (A3 cluster)	*in vivo* intracellular recordings	After odor-reward pairing mainly decreased odor induced activity.	(Grünewald 1999b)

2.3 Functional anatomy of brain areas involved in olfactory learning

The ALs and the MBs are differentially involved in learning, memory formation and retrieval. Figure 1 presents a schematic wiring diagram of the neuronal connections of the olfactory and the putative reward pathway within the honeybee brain.

Antennal lobes

Sensilla on the honeybee antennae comprise the olfactory receptor neurons. Axons of the olfactory receptor neurons enter the brain via four tracts and synapse onto local interneurons (LN) and projection neurons (PNs) within the glomeruli of the AL, the first order neuropil in the olfactory pathway (Suzuki 1975; Mobbs 1982; Arnold et al. 1985; Flanagan et al. 1989; Kirschner et al. 2006). The LNs mediate local information processing within the ALs. The neuronal network in the AL is largely inhibitory, and a subpopulation (approx. 750 out of a total of 4000) of LNs is putatively GABAergic. Consequently, blocking inhibitory synaptic transmission strongly affects odor discrimination and odor-induced spatio-temporal activity (Stopfer et al. 1997; Sachse et al. 2002; Deisig et al. 2010).

PNs transmit olfactory information from the antennal lobes to the lateral protocerebral lobes (lPL) and to the MBs. The PN axons run within two tracts towards the MBs (median and lateral antenno-cerebral tracts: mACT and lACT). Histochemical staining against acetylcholinesterase indicates that acetylcholine (ACh) functions as a neurotransmitter of mACT neurons (Kreissl et al. 1989). The PNs are differentiated into lateral PNs (lPNs) and medial PNs (mPNs) based on their innervation pattern within AL glomeruli. Lateral PNs receive uniglomerular input and innervate the frontally located glomeruli subset T1. Their axons leave the AL via the lACT. Medial PNs innervate the proximally located glomeruli subsets T2-T4 and leave the AL via the mACT. Lateral PNs show down-regulated responses to odor-mixtures but not to their individual compounds (Krofczik et al. 2009). In contrast, mPNs respond to the strongest compound of an odor-mixture and are more temporally sharpened compared to the phasic-tonic responses of the lPNs (Krofczik et al. 2009; Deisig et al. 2010; Rath et al. 2011; Yamagata et al. 2009). These physiological distinctions between PNs are caused by different synaptic input and intrinsic membrane properties. They indicate a differentiated coding of information in time and quality which in turn could induce a more complex processing of memory formation in MB KCs. Furthermore, the dual pathway between the ALs and the MBs – lACT links firstly the AL to the LH and then to the MBs, whereas the mACT firstly innervates the MBs and then the LH – may also lead to advanced processing of olfactory information in the MBs (for a review, see Galizia et al. 2010).

Mushroom bodies

The honeybee MB possesses two separate and asymmetrically shaped calyces, a median and a lateral calyx. Each of the two calyces is again subdivided into three concentric circular compartments, the lip, collar, and basal ring. This architecture represents a common bauplan of the hymenopteran MB (Mobbs 1982; Homberg 1984; Rybak et al. 1993; Strausfeld 2002). In the MB, olfactory information is transmitted to the lip region via the PNs. It converges with other sensory information such as visual, gustatory or mechanosensory input (Erber et al. 1987; Gronenberg 2001) and with input from the VUMmx1 neuron of the subesophageal ganglion (Hammer 1993, see below).

About 170,000 small diameter (Witthöft 1967) KCs form the MB of one hemisphere (Kenyon 1896). Their dendrites build the calyces and their densely packed and parallel axons form the peduncle and the lobes. Large field MB output neurons such as the PE1 neuron (Mobbs 1982) receive synaptic input within the peduncle and the lobes. These output neurons connect the MB to five basic areas of the honeybee brain: (1) the lateral protocerebral lobe, (2) the contralateral MB, (3) the ring neuropil around the α−lobe, (4) the optic tubercle, and (5) the contralateral protocerebrum.
The lateral protocerebral lobe receives antennal input from two sources: directly from the AL via projection neurons and via MB output neurons, such as the PE1 neuron and A4 neurons (Mobbs 1982; Mauelshagen 1993; Rybak et al. 1993, 1998; Strausfeld 2002). Besides output neurons a prominent group of GABA-immunoreactive neurons branches into the output regions of the MB and feedbacks into the calyces (Homberg 1984; Bicker et al. 1985; Erber et al. 1987; Gronenberg 1987; Rybak et al. 1993; Grünewald 1999a; Grünewald 1999b; Ganeshina et al. 2001).

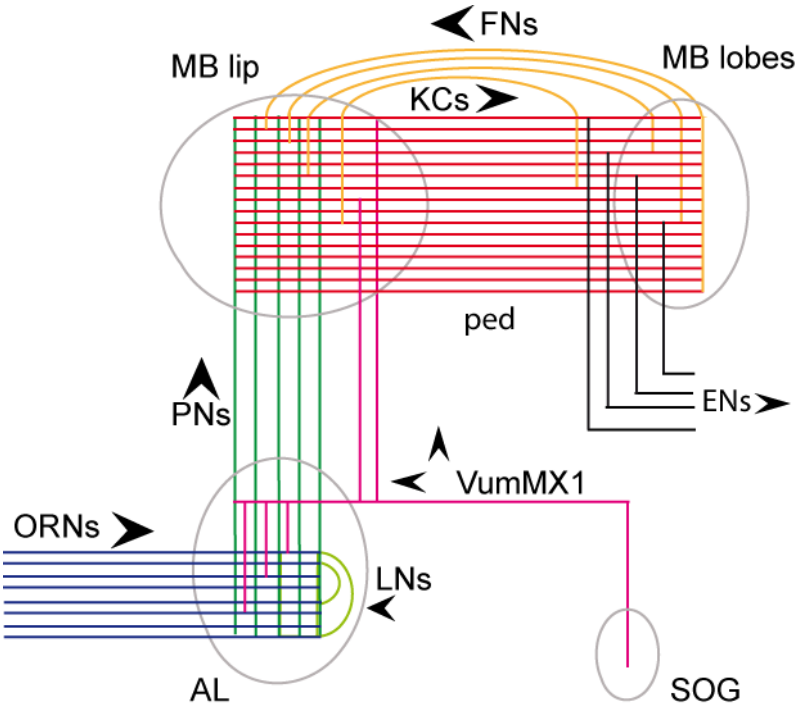

Figure 1 Main neuropils with in- and output regions of the associative olfactory pathway: Olfactory input is provided by axons of olfactory receptor neurons (ORNs) via the antennal nerve. They converge onto local interneurons (LNs) and projection neurons (PNs) of the AL. PNs leave the AL via ACTs to the MB input region – the lip region of the calyces – where they synapse onto KCs. Axons of the MB intrinsic KCs descend via the peduncle (ped) to the MB lobes. MB feedback neurons (FNs) form a feedback loop to the MB calyces where they innervate both KCs and PN axons. Extrinsic neurons receive input typically from numerous KC within the peduncle and the lobes and leave the MB lobes to various protocerebral neuropils. The VUM_{mx1} neuron coming from the suboesophagial ganglion (SOG) represents the reward pathway. Branches of this putative octopaminergic neuron synapse onto AL neurons and MB KCs.

2.4 Physiological bases of classical reward learning

Several lines of evidence indicated that the AL and the MB of honeybees participate in different phases of odor learning and memory formation. (1) Inhibiting neural activity in the AL or the MB causes retrograde amnestic effects (Menzel et al. 1974; Erber et al. 1980). (2) The VUMmx1 neuron innervates the antennal lobes and the MB. Spike activity in the VUMmx1 neuron is sufficient as a substitute for reward during classical conditioning (Hammer 1993). (3) Local injections of octopamine (OA) into the MB or the AL can substitute for the rewarding stimulus during classical

conditioning (Hammer et al. 1998). (4) Odor-induced activity is modified by learning in AL neurons (Faber et al. 1999), MB extrinsic neurons (Mauelshagen 1993; Rybak et al. 1998; Grünewald 1999b; Okada et al. 2007; Haehnel et al. 2010) and KCs (Szyszka et al. 2008; Faber et al. 2001) (4) Prolonged activation of the PKA within the AL induces behavioral LTM (Müller 2000). (6) Learning modulates PKC activity within the ALs and blocking the constitutively active form of the PKC leads to a memory impairment (Grünbaum et al. 1998). (7) Uncaging experiments of glutamate in the MB induced a higher memory rate, whereas uncaging glutamate in the AL showed no effect (Locatelli et al. 2005). (8) Local injections of the anaesthetic procaine into the MB impaired reversal learning (Devaud et al. 2007).

Although these studies identified the MBs and the ALs as important sites for experience-dependent plasticity, the physiological consequences of learning in individual neurons are still a matter of intense research. In a pioneering work Mauelshagen (1993) showed for the first time learning-dependent modulations of odor responses by repeatedly recording from an identified extrinsic neuron of the MB, the PE1 neuron. This neuron receives synaptic input from numerous KCs and specifically decreases its spike frequency during responses to the rewarded CS, but not to the unrewarded CS during differential conditioning. Such a reduction in MB excitation was similarly observed in MB feedback neurons (Grünewald 1999b). Experience-dependent plasticity of MB extrinsic neurons was recently confirmed by extracellular recordings from the MB output areas (Okada et al. 2007; Strube-Bloss et al. 2011) or in imaging studies (Haehnel et al. 2010). The synaptic mechanisms by which MB neurons are modulated are as yet unknown. Menzel et al. (2005) indicate that the synapse between KCs and the PE1 neurons may undergo plastic processes such as long-term potentiation. Okada et al. (2007) argue that either the PE1 neuron receives enhanced inhibition or long-term depression-mechanisms at the KC-PE1 synapse underlie the observed response modulations.

Studies of lPNs and mPNs, revealed learning related plasticity (Yamagata et al. 2009). This leads to the assumption that already in PNs learning-related postsynaptic processes arise in consequence of the existing presynaptic neuronal network in the ALs. The KCs are highly odor-specific and show combinatorial activity patterns like PNs (Szyszka et al. 2008). In particular, KCs have two types of odor processing: sparsening, which may be generated by the inhibitory feedback neurons and

temporal sharpening of postsynaptic KCs responses. This plasticity in KCs may also be the consequence of postsynaptic processing of presynaptic inputs coming from excitatory PNs, inhibitory MB feedback neurons or octopaminergic reward neurons. In conclusion, we assume that in both the AL and the MB learning-related plasticity occurs in the honeybee brain based on the underlying cell physiological events which lead to the described alterations during learning-related Ca^{2+} changes and spike activity in AL PNs and extrinsic MB neurons.

In the honeybee, an identified neuron – the VUMmx1 neuron of the subesophageal ganglion – mediates the reward property of the unconditioned stimulus during classical conditioning (Hammer 1993). Hammer showed that experimentally induced spike activity in the VUMmx1 neuron paired with an odor is sufficient to substitute for the rewarding properties of the unconditioned stimulus. The VUMmx1 neuron has widespread arborizations within the AL, the calyces of the MB and the lateral protocerebral lobe. In these three areas it converges with odor-induced neural activity. The VUMmx1 neuron is probably octopaminergic (Kreissl et al. 1994; Sinakevitch et al. 2005). The widespread distribution of the octopamine receptor AmOA1 in excitatory and inhibitory neurons of the AL and of the MB calyces (Sinakevitch et al. 2011) further supports the hypothesis that in more than one brain region the coincidence between CS and reward is detected. This assumption is confirmed by behavioral analyses. OA injections into the MB or the AL paired with CS stimulations lead to behavioral learning and memory formation (Hammer et al. 1998).

2.5 Transmitter receptors involved in learning and memory formation

The electrical activity of neurons is determined by the gating of ion channels and the respective ionic flow. Several voltage-sensitive membrane currents of KCs and AL neurons were described elsewhere (Grünewald 2012). Since learning is often manifested in an alteration of synaptic transmission of KCs and AL neurons, we focus here on the transmitter-sensitive ionic currents. Honeybee central neurons express nAChR, ionotropic GABA receptors (GABAR) and at least two different glutamate receptors.

Acetylcholine receptors

ACh is the major excitatory transmitter in the nervous system of insects (for review, see Jones et al. 2010). The axons of the ORNs probably release ACh onto postsynaptic neurons within the ALs. AL neurons express functional nAChRs (Barbara et al. 2005; Nauen et al. 2001). In addition, PNs running within the mACT show ACh esterase activity (Kreissl et al. 1989). Honeybee neurons probably express both nicotinic and muscarinic AChRs. Early behavioral pharmacological studies indicated that putative muscarinic antagonists affect the unconditioned and conditioned responses. (review: Gauthier and Grünewald 2012). It was argued that muscarinic AChRs may be involved in learning-dependent behavioral plasticity. However, the honeybee nicotinic receptor is blocked by atropine, which indicates a partially non-nicotinic pharmacology (Wüstenberg and Grünewald 2004). Therefore, muscarinic agents may act partially also via nicotinic receptors in the honeybee brain (at least at higher concentrations as used in earlier studies). In addition, no electrophysiological and pharmacological data on the honeybee muscarinic receptor(s) are available although a gene coding for a muscarinic AChR was described (Hauser et al. 2006).

Nicotinic AChRs are pentameric ligand-gated ion channels and belong to the cys-loop receptor family. Although sequence analyses identified 11 different nAChR subunits in the honeybee genome, Amelα1-9 and Amelβ1-2, the stochiometry of the insect nAChR has not yet been elucidated (for a review, see Jones et al. 2010). In insects, the nAChR insect gene family has seven core groups. These are highly conserved due to their amino acid sequence homology among five studied insect species. In situ hybridisation experiments showed that four nAChR subunits are differently expressed in the honeybee brain during ontogeny (Thany et al. 2003; Thany et al. 2005): The Amelα8, (described as Amelα3 by Thany et al. 2003) is closest to the vertebrate α3 subunit and is found in pupal MB KCs and AL neurons (Jones et al. 2010). The Amelα5 and Amelα7 (originally classified as Apisα7-2 and Apisα7-1, respectively, in Thany et al. 2005) subunits are expressed in both neuropils: MB (KCs type II or clawed KCs) and ALs. The Amelα2 subunit is found in type I and type II KCs, but not in the ALs. The Amelα7 subunit is expressed in type I KCs (Thany et al. 2003).

The physiology and pharmacology of the native honeybee nAChR of KCs and AL neurons was extensively studied *in vitro* (Goldberg et al. 1999; Wüstenberg et al. 2004; Barbara et al. 2005; Barbara et al. 2008). The nAChR is a cation-selective channel with almost equal permeabilities for Na^+ and K^+, and a high Ca^{2+}-permeability (Goldberg et al. 1999). Its pharmacology defines a neuronal nACh receptor profile which shows differences between pupal and adult AL neurons and KCs. The unique expression of the AChR subunit Amelα7 in AL neurons (Dupuis et al. 2011) and alternative expression patterns of the AChR could be responsible for minor differences in ionic current. Honeybee nAChR in pupae/adult AL neurons and KCs are blocked by the nicotinergic blockers curare, methyllycaconitine, dihydroxy-β-erythroidine and mecamylamine. The natural transmitter ACh as well as carbamylcholine are full agonists, whereas nicotine, epibatidine, cytosine and the nenicotinoid imidacloprid are partial agonists.

In olfactory conditioning studies the effects of neonicotinoides on learning and likewise the important roles of the putatively different nAChR subtypes during learning related events were investigated (for a review, see Gauthier et al. 2012). *In vitro* studies, where imidacloprid was applied onto cultured honeybee neurons, show that it acts as a partial receptor agonist and elicits currents similar to those induced by nicotine (Nauen et al. 2001; Deglise et al. 2002; Barbara et al. 2005; Barbara et al. 2008).

Behavioral pharmacological experiments with nAChR antagonists indicated that the nAChRs are involved in learning and memory formation. Injections of the nACh antagonists mecamylamine, α-bungarotoxin or methylylcaconitine into the honeybee brain cause a faster habituation in a non-associative learning paradigm (Gauthier 2010). In an associative learning experiment, the nACh antagonists induced an impairment of memory formation although the affinity of the receptor to imidacloprid is comparably low in honeybees (Cano Lozano et al. 2001; Gauthier 2010).

GABA receptors

Insect GABA receptors are pentameric structures like vertebrate ionotropic GABA receptors (Jones et al. 2006). The activation of honeybee ionotropic GABA receptors induces fast Cl^- currents (Barbara et al. 2005; Barbara et al. 2008; Grünewald et al.

2008). The GABA receptors of honeybee central neurons are probably composed of RDL and LCCH3 receptor subunits with an as yet unknown stochiometry (Grünewald et al. 2008; Dupuis et al. 2010). The native receptor shows a typical insect GABA receptor pharmacology. It is sensitive to picrotoxine, muscimol and CACA, but insensitive to bicuculline. The insecticide fipronil blocks GABA-induced currents (Barbara et al. 2005). Honeybee GABA-induced currents are modulated by intracellular Ca^{2+} (Grünewald et al. 2008). This modulation may be mediated via Ca^{2+}-dependent phosphorylation at one of its multiple phosphorylation sites.

Glutamate receptors

Different glutamate- receptors exist in the honeybee brain: 1. A cation-selective current that is induced by glutamate or AMPA ($GluR_{AMPA}$, Grünewald unpublished). 2. A $GluR_{NMDA}$, which subunits were found in AL neurons and KCs (Zannat el al. 2006; Zachepilo et al. 2008), and which is discussed to be involved in long-term memory (LTM) processes in KCs (Müßig et al. 2010). However, $GluR_{NMDA}$ are still not electrophysiologically described. 3. A chloride current is activated by glutamate applications onto AL neurons ($GluR_{Cl}$; Barbara et al. 2005). The $GluR_{Cl}$ currents comprise of a rapidly-activating, desensitising and a sustained component. This honeybee $GluR_{Cl}$ is partially sensitive to picrotoxin and bicuculline and is blocked by fipronil. It, therefore, shares several properties with the $GluR_{Cl}$ of other insects (for a review, see Cleland 1996). Thus, two independent inhibitory systems within the honeybee ALs may exist: a glutamatergic inhibitory network as well as the GABAergic network (Barbara et al. 2005). Behavioral pharmacological studies generally support this view, since injections of fipronil and ivermectin and co-injections with other glutamate and GABA receptor modulators affect olfactory learning and memory in honeybees (El Hassani et al. 2008, 2009). In addition, honeybee neurons express metabotrobic GluRs which may be involved during memory formation (Kucharski 2007). However, electrophysiological data are missing for that receptor and only the inhibitory $GluR_{Cl}$ of the ALs is implemented in our working model.

Octopamine receptors

Biogenic amines play important roles both in vertebrate and invertebrate nervous systems (Evans 1980; Scheiner et al. 2006). In the honeybee they act as neuromodulators and neurohormones (Mercer et al. 1983; Scheiner et al. 2006).

Reward processing neurons from the SOG (VUM_{mx1} neuron, see above) release OA in the ALs, lateral horn, lateral protocerebrum and in the calyces of the MB (Hammer 1993; Kreissl et al. 1994). One octopamine receptor, AmOA1, has a widespread distribution in the honeybee brain including the AL, MB, central complex, optic lobes and subesophageal ganglion and is also expressed in KCs, in GABA-immunoreactive interneurons of the AL and feedback neurons of the MB (Grohmann et al. 2003; Sinakevitch et al. 2005; Sinakevitch et al. 2011). Probably, multiple OARs are expressed in the honeybee brain. Based on findings of four OAR subtypes in *Drosophila* (Evans et al. 2005), five OAR candidates have been annotated from the honeybee genome (for a review, see Hauser et al. 2006). They are metabotropic, G-protein coupled receptors (Evans 1980). So far, only one receptor, AmOA1R, has been cloned and characterized (Grohmann et al. 2003). Activation of heterologously expressed AmOA1 induces intracellular Ca^{2+} oscillations by applying nanomolar concentrations of OA. In addition, small increases in the concentration of cAMP were observed after applying OA in micromolar concentrations (Grohmann et al. 2003). In another study, micromolar concentrations of OA led to an activation of PKA (Müller 1997).

2.6 Cell integrative model of processes underlying learning related plasticity

Although it is as yet not definitely shown how coincident activation of CS and reward pathways is detected at the cellular level, we integrated the available physiological data into a model. Two candidate cells, KCs and AL neurons, where the cholinergic and octopmainergic pathway converges, may act as cellular coincidence detectors. On one hand this assumption is provided by the expression of the OAR (AmOA1) in the lip region of the MBs clawed KC (Sinakevitch et al. 2011). On the other hand, KCs receive cholinergic input from PNs. KCs express a nAChR (Bicker et al. 1994; Goldberg et al. 1999; Wüstenberg 2004; Deglise et al. 2002; Thany et al. 2005). Hence, we hypothesis the co-expression of at least the OA and nACh receptors in

KCs involved during coincidence detection. Further, we assume the co-expression of GABARs in the same cells. Such a co-expression of OAR and nAChR may be similar found in PNs as like in MB KCs. A model has to explain how the coincidence activation of these receptors leads to learning-related plasticity (Fig.2).

The various intracellular signaling pathways which contribute to the different memory phases in the honeybee brain (for reviews, see Müller et al. 2002; Müller 2012, this issue), are the basis of our cell integrative model of processes underlying learning-related plasticity: (1) The cAMP/PKA cascade plays a key role during the induction of LTM, because blocking the PKA activity during acquisition impairs LTM without affecting short-term memory (STM) or learning (Fiala et al. 1999). (2) OA or stimulation of the bee with the sucrose reward transiently activates the PKA in vivo (Hildebrandt et al. 1995a; Hildebrandt et al. 1995b) or *in vitro* (Müller 1997). (3) The gaseous neurotransmitter NO is required for a stable LTM formation (Müller 1996). It mediates the prolonged PKA activation in the ALs during multiple-trial conditioning (Müller 1996; Müller 2000). (4) Olfactory learning activates a Ca^{2+}- dependent protein kinase C (PKC). Inhibition of the PKC neither affects learning nor STM or LTM, but it impairs a mid-term memory (MTM; (Grünbaum et al. 1998; Müller et al. 2002). Thus, the formation of LTM requires both, NO-dependent prolonged activation of the PKA and protein synthesis. The MTM is induced parallel to the LTM and depends on the constitutive activation of a PKC. Downstream cell physiological (synaptic) events and electrical consequences are less well understood. Probably, reversible covalent modifications of ion channels are involved. In addition, OA may induce Ca^{2+}-regulated pathways (Müller et al. 2002). The question here is: which are the underlying cellular processes of these pathways.

According to our model the CS and the reward pathway activates the nAChR and the OAR. The coincident CS plus reward activation may have two potential intracellular coincidence detectors (Fig. 2): (1) One pathway, which is realized in the MB of the fruitfly *Drosophila melanogaster* (for a review, see Heisenberg 2003) comprises activation of a Ca^{2+} dependent adenylyl-cyclase which induces PKA activation. In the honeybee, the cAMP-dependent PKA, which is activated by an as yet unknown OAR, could be coupled to an adenylyl-cyclase (Eisenhardt 2006). Activation of PKA may phosphorylate the nAChR subunits and thus modulate ACh-induced currents (Himmelreich, unpublished observations). Here, the PKA would act as the CS-reward

coincidence detector. One possible consequence is the phosphorylating of the transcription factor CREB which induces long-lasting learning-related changes (Eisenhardt et al. 2003; Eisenhardt 2006). (2) The other pathway may comprise the activation of the known α-adrenergic like AmOA1 (Evans et al. 2005) in the honeybee coupled to a G_q proteine which leads to an intracellular inositol trisphosphate (IP_3) release. IP_3 itself activates the IP_3 receptors in the endoplasmic reticulum membrane and causes an increase in the cytoplasmatic free Ca^{2+} concentration (Kamikouchi et al. 1998). The activation of the OAR (Wang et al. 2003) potentiated by Ca^{2+} influx through the nAChR on the one hand and by Ca^{2+}-release from the endoplasmic reticulum on the other hand, may also act as a coincidence detector between CS and the reward and may activate unknown Ca^{2+}-dependent kinases. The cytoplasmatic free Ca^{2+} could modulate ACh-induced synaptic currents through the different electrochemical gradients. Alternatively, Ca^{2+}-dependent kinases could phosphorylate the nAChR and thus modulate the ACh-induced currents.

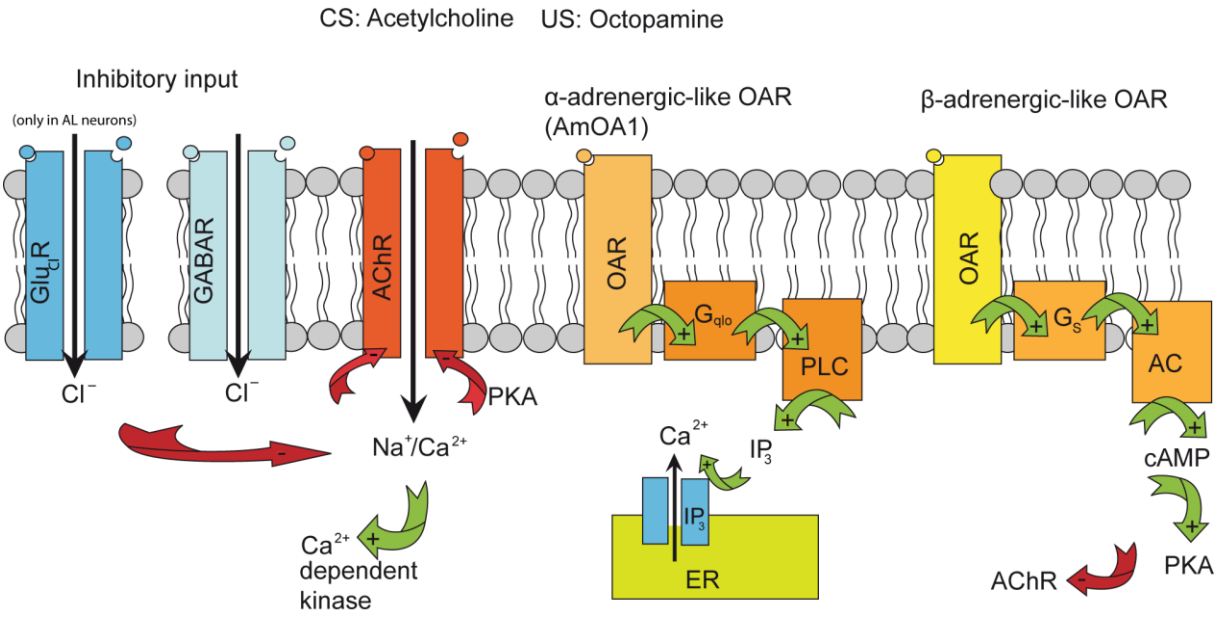

Figure 2: Schematic diagram of cellular physiology in neurons where US and CS pathways converge. Two coincidence detectors may be assumed: (1) The increased intracellular Ca^{2+} level arises from the influx of Na^{+} and Ca^{2+} through the activated nAChR. This represents the olfactory CS pathway. The reward pathway activates either AmOA1 or β-adrenergic-like OAR. The activation of AmOA1 induces a Ca^{2+} release out of internal Ca^{2+} stores (ER = endoplasmatic reticulum). The AmOA1 itself is coupled to a G_q protein and a PLC which increase the concentration of inositol trisphosphate (IP_3). IP_3 in turn binds to the IP_3 receptor in the ER membrane which induces a Ca^{2+} release. This may modulate the ACh-induced synaptic currents. The elevated cytosolic Ca^{2+} level could also activate as yet unknown Ca^{2+}-dependent kinases which may phosphorylate the nAChR and thus modulate ACh-induced currents. (2) The other coincidence detector could be the cAMP-dependent PKA which may phosphorylate the nAChR and modulate the ACh-induced currents. This pathway is elicited by the activation of β-adrenergic-like OARs (which are identified but not yet characterized in the honeybee). It is assumed that they are coupled to adenylyl-cyclases (AC). The activated AC induces formation of cAMP and activation of PKA. Inhibitory input is provided by GABA- and glutamate (only in the ALs, indicated by the interrupted membrane) induced Cl^{-} influx which balances the membrane depolarization, induced by ACh-evoked Na^{+} and Ca^{2+} currents. (-/+ arrows= indicating negative or positive influence of the indicated molecule/receptor).

Influx of Cl^{-} through GABA-/Glu-gated channels changes the intracellular electrochemical gradient which reduce the CS induced membrane depolarisation and intracellular Ca^{2+} influx. Fast inhibitory inputs from processes of MB feedback

neurons elicit GABA-induced fast Cl^- currents which may be responsible for spike sparsening and temporal sharpening of KCs´ odor responses (Szyszka et al. 2008). pathways.

Additionally, our model partially explains effects of insecticides or acaricides, on cognitive behavior. Several of these substances interact with the nAChR or with the acetylcholine esterase (neonicotinoids/ organo phosphates, reviewed by Thany 2010 and Decourtye et al. 2010). Amitraz or thymol modulate the OAR (reviewed by Blenau et al. 2011, this issue). Thymol also interact with a tyramine receptor (Enan 2005) or at higher concentrations with the GABA receptor (Priestley et al. 2003). Fipronil impairs the honeybee GABAR (reviewed by Belzunces et al. 2012, this issue). Thus these substances interfere with putative synaptic events underlying learning-related coincidence detection mechanisms.

Finally, based on the similar synaptic inhibitory, excitatory and octopaminergic input and the similar postsynaptic receptor distribution in antennal lobes and mushroom bodies (as described above), our model may therefore apply to projection neurons and intrinsic Kenyon cells. It serves as a working hypothesis, and one challenge for the future is to identify the similarities and differences between learning-induced plasticity in antennal lobe neurons and Kenyon cells and their behavioral relevance.

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CHAPTER 3[2]

3 Octopamine and serotonin as neuromodulators in cultured honeybee mushroom body neurons and antennal lobe neurons

3.1 Abstract

The biogenic amine octopamine (OA) plays a crucial role in the honeybee brain and is reported to be the neurotransmitter of the reward system during appetitive olfactory conditioning. Many learning models include OA as an important part in the engram of learning and memory formation. But less is known about OA-evoked Ca^{2+} responses in the neurons where learning and memory formation takes place. Serotonin, another biogenic amine, has a widespread distribution in the honeybee brain; serotonin receptors are also found in the neuron classes where learning and memory formation is reported. Despite the unclear role of serotonin in learning, we investigated OA- and serotonin-evoked Ca^{2+} responses in Kenyon cells (KCs) and antennal lobe neurons, two neuron classes of neuropils which are mainly responsible for learning and memory formation in the honeybee. Further, we demonstrate OA-induced modulatory effects on acetylcholine-induced Ca^{2+} signals and inward currents in KCs and antennal lobe neurons. On the cellular level, we show an interaction of the neurotransmitters OA and acetylcholine (ACh). These findings suggest that the conditioned stimulus (CS) and unconditioned stimulus (US) during olfactory conditioning, represented in our study by ACh- and OA-receptor activation, respectively, converge on the same neurons of the antennal lobes or mushroom bodies and lead to intracellular modulations.

[2]This chapter is based on the following manuscript: “Octopamine and serotonin as neuromodulators in cultured honeybee mushroom body and antennal lobe neurons”, by Himmelreich S, Grünewald B

Experiments and data analysis

All experiments were designed and performed by Sophie Ziegler-Himmelreich. All data analysis was done by Sophie Ziegler-Himmelreich.

Writing process and figure designing

Sophie Ziegler-Himmelreich wrote the whole manuscript and designed and developed all figures.

Bernd Grünewald provided suggestions and criticisms on parts of the text.

3.2 Introduction

The honeybee, with its behavioral richness compared to simpler organisms, is a valuable model for studying learning and memory and their underlying cellular mechanisms. Kuwabara (1957) and Menzel et al. (1974) reported that the honeybee reflexively extends its proboscis after a sucrose stimulus is presented to the tarsi or to the antennae. This simple reflex can be conditioned to olfactory stimuli. Based on these findings, much is known today about learning-related neuronal structures and their underlying cellular mechanisms in the honeybee brain. However, not much is yet known about the cellular mechanisms involved in the convergence of the CS and US during olfactory learning in the honeybee brain.

Several honeybee learning models hypothesize possible neuronal sites and cellular mechanisms during the convergence of the CS and US in the honeybee brain (for reviews, see Himmelreich and Grünewald 2012, Menzel 2012, Eisenhardt 2006, Müller 2002). Important studies of *Drosophila* have already provided more insight into the location and the cellular mechanisms involved in the coincidence detection of CS and US during appetitive or aversive conditioning (Ueno et al. 2012, Tomchik and Davis 2009, Gervasi et al. 2010). Ueno et al. demonstrated that the simultaneous stimulation of the antennal lobe and the ventral nerve cord, which conveys shock signals, establishes a long-term enhancement of Ca^{2+} responses, specifically at synapses which connect the antennal lobe with the mushroom body. Tomchik and Davis showed that in mushroom bodies, the temporal pairings of the reward or punishment transmitters, OA and dopamine and the neuronal depolarization through ACh applications produce subadditive or synergistic effects on the intracellular cAMP ($[cAMP]_i$), respectively. The synergistic elevation of $[cAMP]_i$ induced by dopamine is dependent on the activity of rutabaga adenylyl cyclase (*rut* AC, adenylylcyclase 1), which is assumed to be activated by a previous intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The $[Ca^{2+}]_i$ increase is caused by the Ca^{2+}-influx through the nicotinic ACh receptor (nAChR) into the cell lumen and through the activation of a G_s-protein-coupled dopamine receptor. The synergetic elevation of cAMP-activated protein kinase A (PKA) was also found during co-stimulations of ACh with either dopamine or OA in *Drosophila* MBs, caused by the activity of *rut* AC (Gervarsi et al. 2010). These findings and several studies in other systems point to the *rut* AC or to similar adenylyl cyclases functioning as a molecular coincidence detector during learning (in *Aplysia*:

Abrams et al. 1991, in mice: Wu et al. 1995, for review Wang and Storm 2003). So far, neither evidence has been found for a similar coincidence detection in honeybees, nor evidence has been reported for the interaction of the CS-US convergence on the cellular level in honeybee neurons. In the present work we examine the possible convergence of the CS and US by investigating the effects of OA on the nAChR in primary neuron culture.

In the honeybee, the main excitatory transmitter of the olfactory pathway is ACh (reviewed by Thany 2010). The olfactory receptor neurons of the antennae form synapses within the antennal lobe, where ACh is released. Cholinergic synapses are also found in the lip region of the mushroom body calyces, where distinct projection neurons (PNs), a subpopulation of antennal lobe neurons, synapse onto KCs (Kreissl and Bicker 1989). *In vitro* studies of cultured antennal lobe neurons and KCs show the common physiology and pharmacology of the cation-selective nAChR (Goldberg et al. 1999, Déglise et al. 2002; Wüstenberg and Grünewald 2004; Barbara et al. 2005, 2008). The nAChR in KCs has almost equal permeabilities for Na^+ and K^+ as well as a high Ca^{2+}-permeability (Goldberg et al.1999). In our hypothetical model of the cellular mechanisms underlying olfactory conditioning, the activation of the nAChR within the olfactory pathway represents the CS during olfactory conditioning (see Figure 6).

In the honeybee, the transmitter of the reward is thought to be the biogenic amine OA, which is released by the reward processing neuron VUMmx1 (the ventral unpaired median neuron 1 of the maxillary neuromere). It originates in the subesophageal ganglion (SEG) and has dendritic arborizations into the antennal lobes, the lateral horn, the lateral protocerebrum, and the mushroom bodies (review Perry and Barron 2012). There is important evidence indicating that OA mediates the reward signaling in honeybees: 1. The depolarizations of the VUMmx1 neuron during olfactory conditioning lead to behavioral learning (Hammer 1993); 2. Injections of OA either into the mushroom body calyces or into the antennal lobes paired with presentation of the odor as the CS was sufficient as a substitute for the sucrose reward during olfactory conditioning (Hammer and Menzel 1998); 3. The depletion of biogenic amines by reserpine impaired olfactory conditioning. OA injections into the brain rescued conditioning in reserpinized bees. Today, five candidates of G-protein-coupled OA receptors have been annotated from the honeybee genome (for review,

see: Hauser et al. 2006). The α-adrenergic-like AmOA1 receptor has been molecularly and functionally characterized (Grohmann et al. 2003). It is coupled to a G_q-protein and mediates the hydrolysis of inositol phosphates and a subsequent increase in cytosolic Ca^{2+} levels. The AmOA1 receptor is found in the antennal lobes and mushroom bodies besides many other brain areas. Here, GABA-immunoreactive interneurons of the antennal lobes, KCs, and feedback neurons of the mushroom bodies express the AmOA1 receptor (Sinakevitch et al. 2011). The existence of an not yet identified β-adrenergic-like OA receptor, possibly coupled to a G_s-protein and a adenylyl cyclase, has been indicated in experiments in which OA is applied to cultured KCs and to the antennal lobe which induces PKA activity (Hildebrandt and Müller 1995; Müller 1997). In *Drosophila* also a cAMP/PKA pathway is indirectly activated by a $[Ca^{2+}]_i$ increase which is induced by possible G_q-protein coupled receptors. The $[Ca^{2+}]_i$ increase lead to the activation of the *rut* AC and therefore to a cAMP/PKA pathway. Interestingly, the supposition of a secondary reaction of the G_q-protein coupled OA receptor, AmOA1, leading to an activation of the cAMP/PKA pathway by activation of an adenylyl cyclase No 1 (AC1), similar to the *rut* AC in *Drosophila*, has not yet been shown in honeybees: No PKA activity could be found after raising the intracellular $[Ca^{2+}]_i$ neither after applications of ACh (strong Ca^{2+} influx) nor after applications of the Ca^{2+}-ionophore A21187 or KCl (Müller 1997). A newer study described another similar *rut* AC in honeybees, the AmAC8, which was found intensively expressed in mushroom body neurons and showed a Ca^{2+}/calmodulin-dependent activity in heterologously expressed HEK cells. However, no study so far has shown a possible relation between AmOA1 receptor activation and AmAC8 activity. Furthermore, little is known about the activation profiles of the α-adrenergic-like OA receptors which lead to a $[Ca^{2+}]_i$ increase, neither in KCs nor in antennal lobe neurons. The study of heterologously expressed AmOA1 receptors in HEK cells shows strongly oscillating intracellular Ca^{2+}-transients after OA applications (Grohmann et al. 2003). Another study examined native OA receptors in brain homogenates and their pharmacological properties (Degen et al. 2000). But so far, no study has shown OA-induced Ca^{2+}-transients in KCs or antennal lobe neurons themselves, although the OA receptor plays a crucial role in most models of CS and US convergence in the honeybee.

The role of serotonin (5-hydroxytryptamine, 5HT) during learning processes is unclear so far, even though a widespread distribution of different serotonin receptors within neuropils associated with learning and memory are found in the honeybee brain (Thamm et al. 2010, Schlenstedt et al. 2006). In addition to learning processes, many different behavioral functions of the serotonergic system are reported in *Drosophila* and the honeybee (for review Blenau et al. 2013). Only a few studies have investigated the role of serotonin during learning and memory. In the honeybee, injecting serotonin into the brain leads to the impairment of olfactory learning (Müller 1997), which may indicate the activation of a serotonin receptor inhibiting the cAMP/PKA pathway, like the molecular and functionally described 5HT1A receptor (Thamm et al. 2010). Another study reported that serotonin could mediate the association of odors with the malaise caused by ingesting amygdalin (Wright 2011). Recently, a 5HT1A receptor (Thamm et al. 2010), a 5HT7 receptor (Schlenstedt et al. 2006) and two 5HT2 receptors (Thamm et al. pers. comm.) of the honeybee have been functionally characterized after heterologous expression in HEK cells. The 5HT2α and 5HT2β receptors couple to G_q-proteins and should lead to an increase in intracellular Ca^{2+}, resembling the signaling pathway of the AmOA1 receptor (Thamm et al. pers. comm.).

We examined for the first time OA- and serotonin-induced Ca^{2+} transients in KCs and antennal lobe neurons in primary neuron culture. The patterns of OA- and serotonin-induced Ca^{2+} transients in KCs and antennal lobe neurons vary according to their neuropil origin. The findings of biogenic amine-induced Ca^{2+} transients led us to question whether these transients could interact with or modify ACh-induced Ca^{2+} signals or inward currents. We tested this hypothesis in the second part of our study. Here, we show a decrease in ACh-induced Ca^{2+} transients after the application of OA during Ca^{2+} imaging experiments. Patch-clamp recordings also show a decreased ACh-induced inward current after applications of OA as well as of serotonin, indicating the two biogenic amines have a modulatory effect on the nAChR through intracellular pathways. For the first time, our study indicates a modulatory interaction between the reward transmitter OA and ACh, the olfactory pathway representing transmitter. Therefore, our study may also be the first to show cellular mechanisms during convergence of the CS- and US-activating pathways on the receptor level as

shown in the hypothetical model of cellular mechanisms underlying olfactory conditioning (Figure 6).

3.3 Materials and methods

Primary neuron culture

The Kenyon cells and antennal lobe neurons of honeybee (*Apis mellifera carnica*) pupae of the pupal stages P6-7 were dissected following a modified version of a protocol originally developed by Kreissl and Bicker (1992). After the mushroom bodies or the antennal lobes were dissected, the neuropils were transferred into a calcium-free solution for the first gentle dissociation of the tissue (mM: 130 NaCl, 5 KCl, 10 $MgCl_2$, 25 glucose, 180 sucrose, and 10 Hepes; pH 7.2) and then transferred into a preparation medium (Leibovitz L15 medium, Gibco BRL, supplemented with 22.2 mM sucrose, 22.2 mM fructose, 0.09 mM glucose, 0.029 mM proline M, 0.5 ml/l penicillin/streptomycin, 50 µl/l gentamycin, 500 ± 10 mOsmol l^{-1}). Gentle trituration with a pipette dissociated the cells completely. Thereafter, the cells were allowed to adhere to a glass-bottom dish coated with Concavalin A (20 µl cell suspension per dish). After 45 min, the cells were floated with 2 ml of culture medium (containing Leibovitz L15 medium, see protocol above, and supplemented with 2 mM Pipes, 14.9% FCS and 1.2% yeastolate) and were kept at 26°C in an incubator. The experiments were performed 2-4 days after dissection.

Fluorometric calcium measurements

The intracellular Ca^{2+} concentrations were measured with the ratiometric indicator dye FURA-2 (0.5 µM), which was associated to an AM ester for membrane permeability. The dye was solved in standard saline solution (in mM: 130 NaCl, 6 KCl, 4 $MgCl_2$, 5 $CaCl_2$, 10 Hepes, 25 glucose, 170 sucrose, 500 ± 10 mOsmol l^{-1}, pH 6.7). During FURA-2 loading, the cells were kept in the dark for 30 min. Thereafter, standard saline solution was applied to the cells to wash out extracellular FURA-2, and subsequently, the cells were allowed to rest for 10 min to ensure that the cells returned to their resting states.

After the dye loading procedure was completed, the cell dish was mounted on the stage of an inverse Zeiss microscope with a 20x/0.4 oil immersion objective (Zeiss, Oberkochen/Jena, Germany). The imaging setup consisted of a charge-coupled device camera (CCD camera, Till Imago) and a Polychromator V, which were both controlled by the software Till Vision version 4.5 (all from TILL Photonics GmbH, Munich, Germany) running under Windows. The FURA-2 fluorescence intensities were calculated by dividing the emission intensities (at 520 nm) measured during excitation wavelengths of 340 nm (F^{340}, exposure time 5 ms) and 380 nm (F^{380}, exposure time 20 ms). Frames were taken in 5-10 Hz intervals. Data were acquired using 4 x 4 on-chip binning for the measurements and were subsequently analyzed as 12-bit grayscale images. The cells were defined as regions of interest (ROIs) from the center of the cell bodies and the grayscale values of the fluorescence intensities were transformed into false color-coded images for visualization. The fluorescence intensity ratios were converted into relative changes (ΔF/F), where F was measured as the average of frames 1-50 or 1-100 (depending on the protocol and sampling rate) before stimulus onset. Statistics and data analysis were performed in MATLAB (Version 2012a, MathWorks, Natick, MA, USA).

Electrophysiology

Patch clamp experiments were performed in a whole-cell configuration mode with a gigaohm seal while following the methods described by Hamill et al. 1981. Recordings were performed with an EPC9 amplifier (HEKA Electronik, Lamprecht, Germany), which was controlled by the software Patch-Master Version 2.4 (Heka Electronik). The offset correction and the correction of the capacitance currents caused by the pipette were corrected using the amplifier's automatic mode "c-slow" and the whole-cell capacitance was corrected with the amplifier's "c-fast" mode. The data was low-pass filtered at 2 kHz with a four-pole Bessel filter. The glass pipette was made of borosilicate (GB150-8P; Science Products GmbH, Hofheim, Germany) with a resistance of 6-8 MΩ for antennal-lobe neurons and 8-10 MΩ for KCs. The glass pipette was filled with an internal standard solution containing (in mM): 115 potassium gluconate, 40 KF, 20 KCl, 4 $MgCl_2$, 5 BAPTA tetrapotassium salt,

3 Na_2ATP, 0.1 Na_2GTP, 6 gluthatione, 150 sucrose, and 10 HEPES-Bis-tris; pH 6.7, 490 ± 10 mOsmol l^{-1}).

Transmitter application

For calcium imaging and patch-clamp recordings, a custom-made four-barrel pipette was used for transmitter application. The glass pipette was fashioned from borosilicate glass (GC100F-10; Harvard Apparatus, Edenbridge, UK) and pulled from a vertical puller (Model 700C; David Kopf Instruments, US) with an opening for each barrel measuring 0.5-1 µm in diameter. For calcium imaging and patch-clamp recordings, the pipette was placed next to the cells, no closer than 40-50 µm. The pipette was connected with an air pressure system (npi electronic GmbH, Tamm, Germany) which was controlled by the imaging software Till Vision (TILL Photonics) during Ca^{2+}-imaging experiments or by Patch Master (HEKA Elektronik) during patch-clamp recordings. The cells were continuously superfused with external standard saline at approx. 7.2 ml / min (bath exchange in ~2 s).

3.4 Results

Octopamine- and serotonin-induced Ca^{2+} transients

The signal pattern after the application of 1 µM OA or 1 µM serotonin in KCs and antennal lobe neurons varies significantly between both cell types within 150 s after OA application (Barnard's Exact Probability Test: p = 0.019, KCs N: 85, antennal lobe neurons N: 37) and after serotonin application (Barnard's Exact Probability Test: p< 0.001, KCs N: 60, antennal lobe neurons N = 18; see Figure 1 *B2*). Some representative Ca^{2+} signals after the application of 1 µM OA in KCs and 1 µM serotonin in antennal lobe neurons are shown in Figure 1 *B1*. The majority of KCs express Ca^{2+} signals with an oscillating character after OA application (85% of the cells) and serotonin (88%), whereas the antennal lobe neurons respond similarly to OA and serotonin with oscillating and slowly increasing Ca^{2+} signals (64% and 50%, respectively). The KCs express Ca^{2+} signals which increase slightly over a duration of 100 s without any transmitter application, as shown in Figure 1 C. We chose to

use a time period of 33 s after OA application in modulation protocols (see Figure 3) because at this time, the double SEM of the mean Ca^{2+} signals after saline applications (SEM in light gray, Figure 1 C) clearly differentiated from the double SEM of the mean Ca^{2+} signals after 1 µM OA application (SEM in dark gray). The application of OA receptor antagonists, 100 µM mianserin and 100 µM epinastin, blocked the Ca^{2+} signals induced by 1 µM OA (Figure 1 D). Interestingly, serotonin-induced Ca^{2+} signals were also blocked by mianserin and epinastin, indicating an antagonistic impact of mianserin and epinastin on the serotonin receptor that has not yet been previously shown.

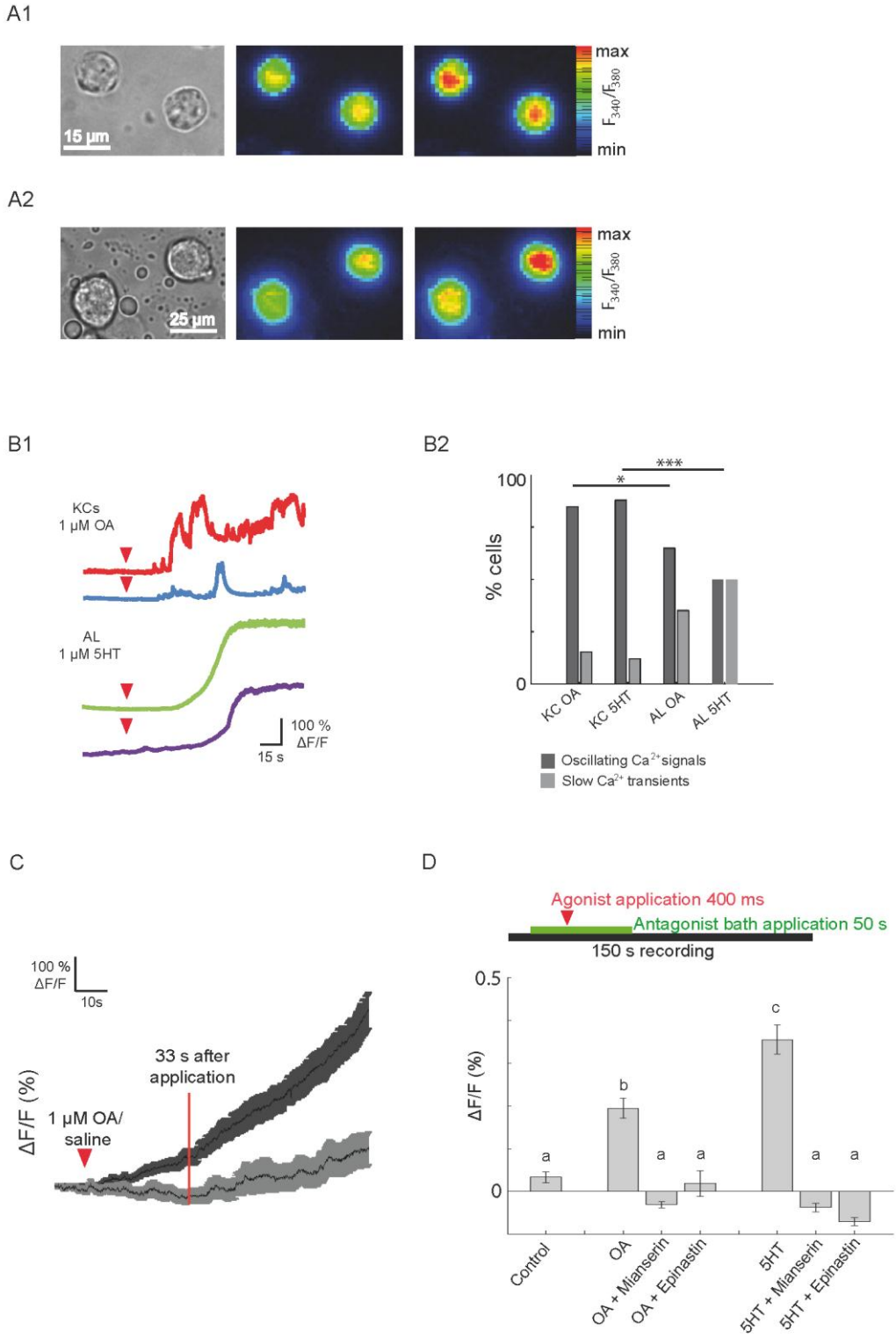

Figure 1 | Ca^{2+} signals induced by OA and serotonin in KCs and antennal lobe neurons. *A* Pupal KCs (*A1*) and antennal lobe neurons (*A2*) loaded with calcium-indicator dye FURA-2. In the images on the left, neurons are shown in bright-field

illumination. In the middle and on the right, neurons are shown in false color before and after OA application, respectively. *B1* depicts examples of two different types of Ca^{2+} transients recorded for 150 s after 1 µM OA application (400 ms, indicated by small red arrows) through an application pipette. The first two traces represent typical Ca^{2+} transients with an oscillating character in KCs, the latter two represent typical slowly increasing Ca^{2+} transients in antennal lobe neurons. In *B2*, the KCs express significantly more oscillating Ca^{2+} transients after OA and serotonin applications compared to antennal lobe neurons (Barnard's Exact Probability Test: KCOA-ALOA $p = 0.019$, KC5HT-AL5HT $p < 0.001$; N: KCOA 85, KC5HT 58, ALOA 37; AL5HT 18). In *C*, the mean Ca^{2+} signal and the SEM (dark gray) of 85 KCs after 1 µM OA application is shown with the mean Ca^{2+} signal and the SEM (light gray) of 30 KCs after the application of the control solution (standard saline). For further experiments, a time point was chosen where the double SEM of both curves had no overlay (33 s after saline/agonist application, indicated by the red line). *D* shows the mean Ca^{2+} signal and the SEM during a time interval of 50 s during antagonist application via bath (100 µM mianserin and 100 µM epinastin, see application protocol in the diagram) compared to a single agonist application, 1 µM OA and 1 µM serotonin or saline application (serving as a control), all applied through an application pipette for 400 ms. A significant reduction in Ca^{2+} transients is found after the application of OA receptor antagonists; the antagonists also significantly block serotonin-induced Ca^{2+} transients (ANOVA followed by a Bonferroni post-hoc test: control (N = 30) to OA (N = 85): $p \leq 0.01$; control to 5HT (N = 66): $p \leq 0.001$; OA to OA + mianserin (N = 54): $p \leq 0.001$; OA to OA + epinastin (N = 18): $p \leq 0.01$; 5HT to 5HT + mianserin (N = 14): $p \leq 0.001$; 5HT to 5HT + epinastin (N = 5): $p \leq 0.001$. Significant differences are indicated by different letters above the bars.

Analysis of the half-life of Ca^{2+} signals

To analyze the half-life of Ca^{2+} signals having an oscillating character, the underlying slow increase of the Ca^{2+} signals occurring during the 150-s recording time had to be shifted to the zero baseline. To accomplish this, all the minima occurring during a Ca^{2+} response were fitted. This fitting procedure resulted in a fit curve which was then subtracted from the experimental data in a second step, thereby shifting the experimental data during an entire Ca^{2+} response to the zero baseline as shown in Figure 2 *A*. These shifted data were the basis for following exponential fittings of the decaying Ca^{2+} signals and the calculation of the half-lives of the decays as shown in Figure 2 *B1*. Highly significant differences in the half-lives of Ca^{2+} decays were found between KCs and antennal lobe neurons after OA applications (Figure 2 *B2*). Here, the KCs' Ca^{2+} signals decay faster than the signals in antennal lobe neurons after OA

applications. Interestingly, the Ca^{2+} signals decrease significantly more rapidly after OA applications than after serotonin applications in KCs (Figure 2 *B2*).

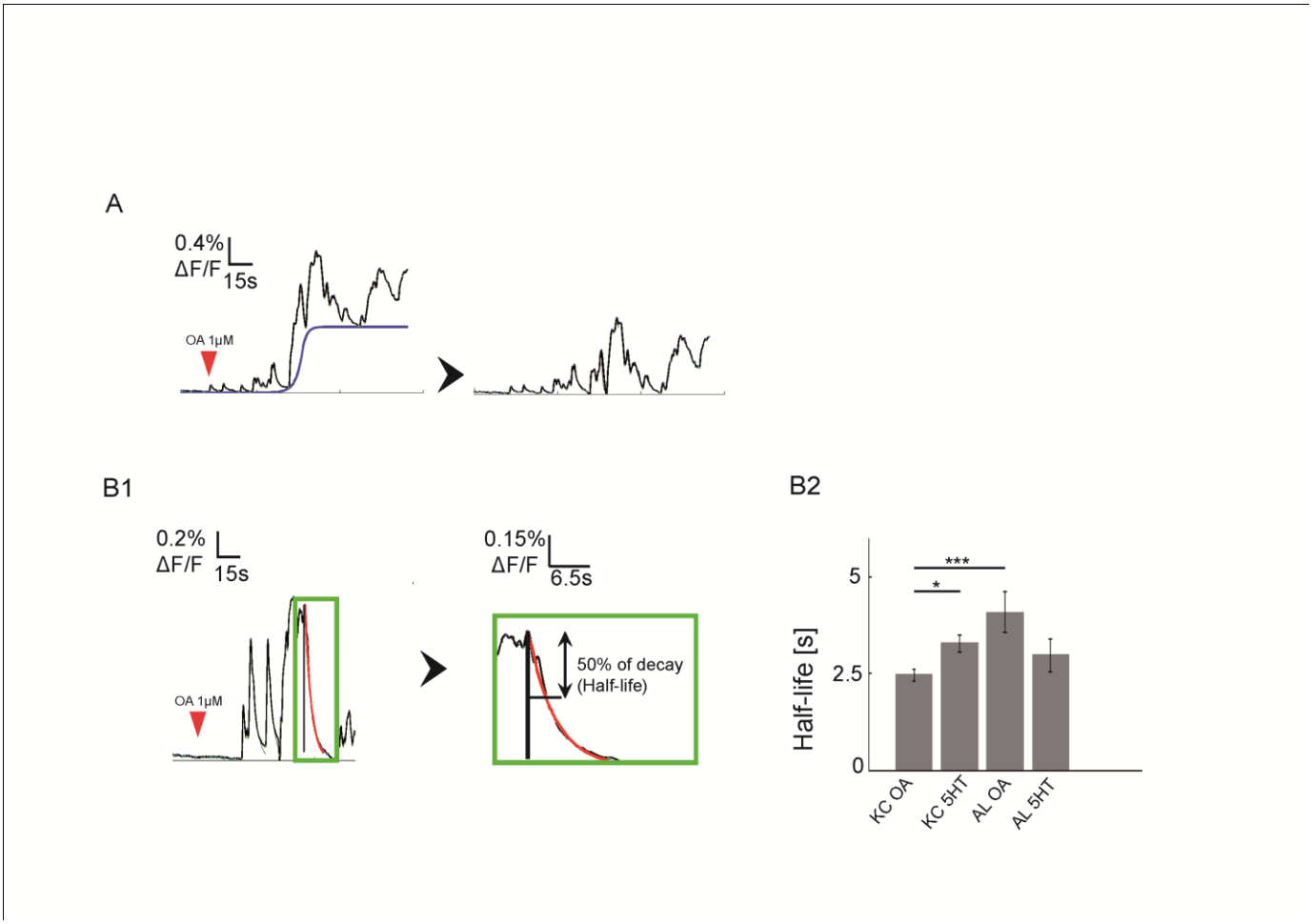

Figure 2 | Different Ca^{2+} signal decays in KCs and antennal lobe neurons after OA and serotonin applications. The analysis of the decay of individual Ca^{2+} signals after agonist application is depicted in Figure2. *A* shows a Ca^{2+} transient with an oscillating character together with a fitting of the signal's minima (in blue). The fit-curve was subtracted from the experimental data, yielding a shift to the zero baseline as the basis for ongoing exponential fittings. *B1* shows the Ca^{2+} response to a 1-µM OA application and the magnification of a single decaying Ca^{2+} signal and its exponential fitting (in red) for the calculation of its decay half-life. In *B2*, the half-life exponential decays were calculated for each Ca^{2+} response induced by OA and serotonin in the KCs and antennal lobe neurons. Hence, Ca^{2+} responses with an oscillating character of one cell have several Ca^{2+} signal decays and their half-life values were averaged in each cell. The most significant differences regarding the decay of the Ca^{2+} signals are found between the KCs (KC OA: N = 72; KC 5HT: N = 58) and the antennal lobe neurons (AL OA: N = 24; AL 5HT: N = 7) after OA application (ANOVA followed by a Tukey's least significant difference post-hoc test: $p < 0.001$). Significant differences were also found between the decay times between the OA- and serotonin-induced Ca^{2+} responses (ANOVA followed by a Tukey's least

significant difference post-hoc test: $p \leq 0.05$). The decays of Ca^{2+} signals in KCs are slower after serotonin application than after OA application.

Modulatory effects of ACh-induced Ca^{2+} signals and inward currents

Three different protocols of ACh applications, each with differing values for the amount of ACh applied and for the sample rate, were tested. Two protocols have a negative influence on the ACh-induced Ca^{2+} signal, indicated by decreasing Ca^{2+} peaks during the recording time (Figure 3).

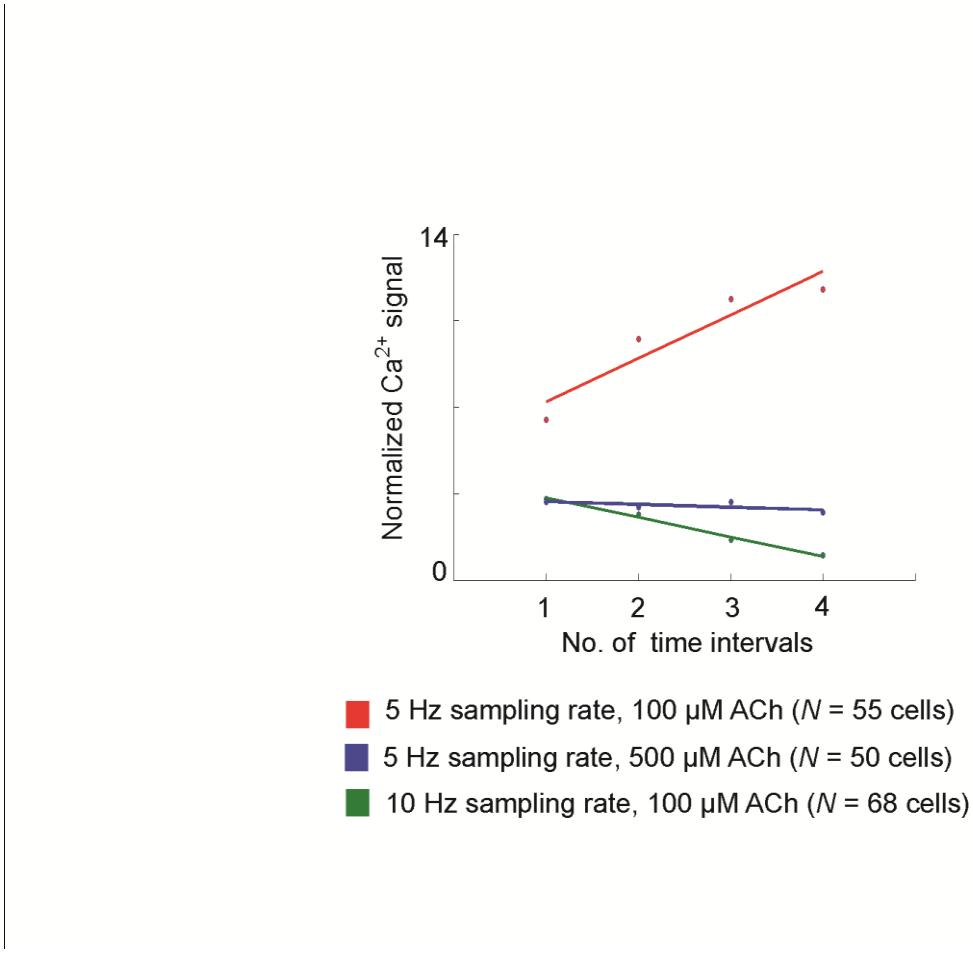

Figure 3 | Transmitter concentrations and sampling rate during Ca^{2+} imaging experiments influence the Ca^{2+} signal. Each median of the normalized ACh-induced Ca^{2+} signals for a given 45-s measurement interval is presented by a dot; the time intervals are labeled 1-4 in order of increasing time. Any increase or decrease in the Ca^{2+} signals is indicated with a linear regression for better visibility. Here, the three different protocols are color coded: 1. Red: sampling rate 5 Hz, 100 µM ACh; 2. Blue: sampling rate 5 Hz, 500 µM ACh; Green: sampling rate 10 Hz, 100 µM ACh. The first protocol is the only one that results in increasing Ca^{2+} signals

during measurement. For our subsequent experiments of OA-induced modulatory effects, we used the first protocol to avoid unpredictable “run-down” effects during measurements (for explanations of “run-down” effects see text below).

Compared to other protocols, the experimental protocol shown in Figure 4 *A1* has no decreasing effect on the Ca^{2+} signal: With four consecutive ACh applications for 400 ms through the application pipette with an application interval of 45 s to avoid desensitization of the nAChR and an image-sampling rate of 5 Hz, the KCs induce strong Ca^{2+} signals after 100-µM ACh applications (Figure 4 *A1*). The other two protocols shown in Figure 3 may show “run-down” effects, which could be caused by too high amounts of ACh and therefore by longer receptor desensitization or by bleaching effects of the FURA molecule after higher sampling rates. The protocol without decreasing effects served as a control and as a basis for the protocol of experiments with 1-µM OA applications (the groups are named A1 and A2, respectively, Figure 4 *A1* and 4 *A2*). In both groups, the peak of the Ca^{2+} signal after the first ACh application was used as the normalization factor for two consecutive ACh-induced Ca^{2+} signals (Figure 4 *A*, indicated in purple and green, respectively). Neither of the two experimental groups showed a significant difference in the Ca^{2+} peak of the first ACh-induced Ca^{2+} signal before normalization, as shown in Figure 3 *C* (Wilcoxon rank sum test; $p = 0.069$, $N = 29$); nor do the peaks of the normalized Ca^{2+} signals after the third and fourth ACh applications differ significantly (Figure 4 *D*; A1 (3) ACh and A1 (4) ACh: Wilcoxon rank sum test, $p = 0.106$, $N= 29$). Highly significant differences were found between the ACh-induced peaks of normalized Ca^{2+} signals after OA application (Figure 4 *D*; A2 (3) ACh and A2 (4) ACh: Wilcoxon rank sum test, $p = 0.001$, $N = 29$). The same highly significant differences were found between the decreased peaks of the normalized Ca^{2+} signals after OA application and the peaks of Ca^{2+} signals of the control experiment (Figure 4 *D*; A1 (3) ACh compared to A2 (3) ACh/ A1 (4) ACh compared to A2 (4) ACh: Wilcoxon rank sum test, $p < 0.001$, each group $N = 29$).

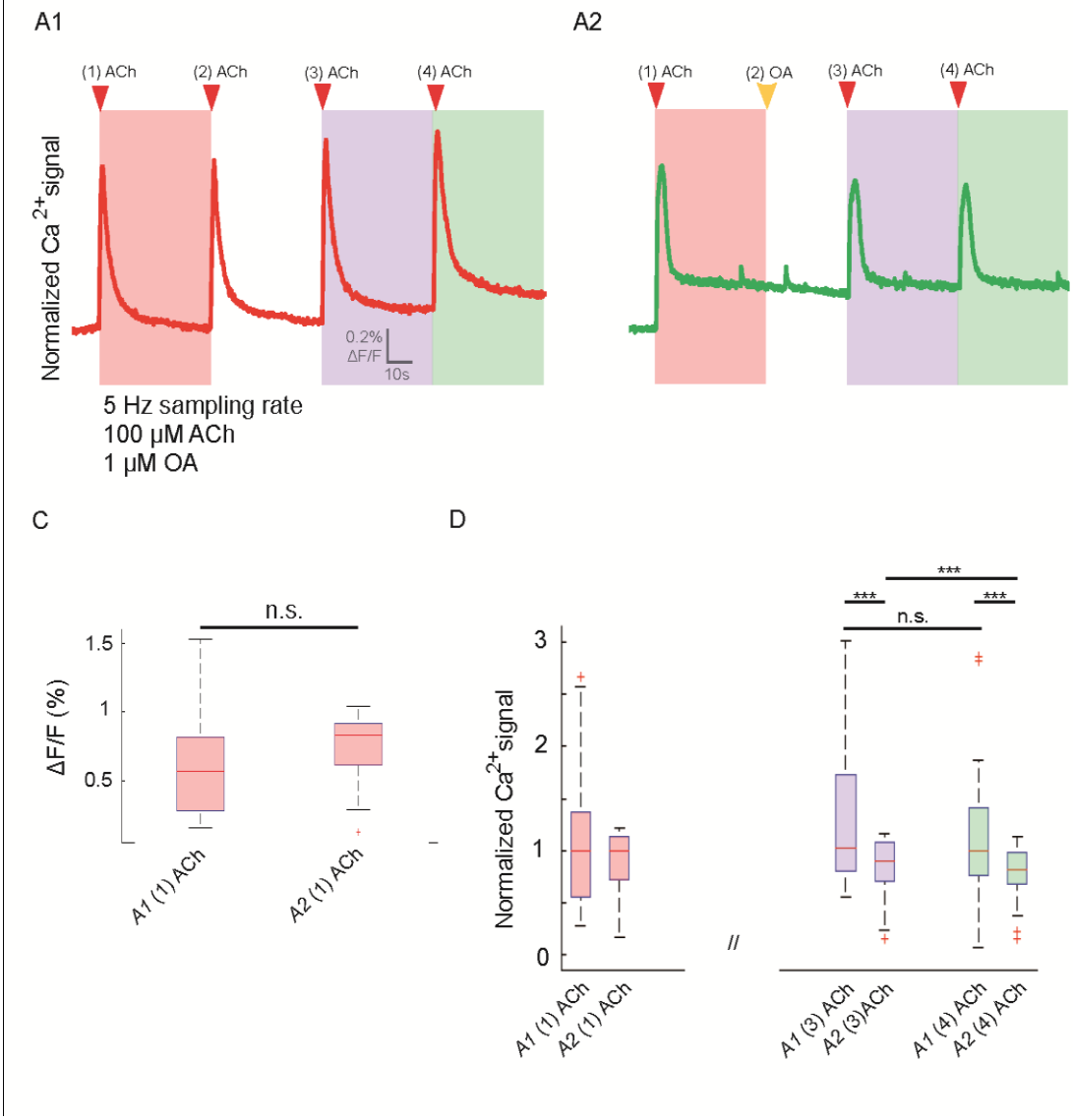

Figure 4 | ACh-induced Ca^{2+} signals decrease after OA application in KCs. Figure 4 *A* shows representative Ca^{2+} signals after 100 µM ACh are applied to KCs (5-Hz sample rate, 191-s recording duration). *A1* shows a protocol of 4 consecutive ACh applications within a 45 s time interval. In *A2*, 1 µM OA was applied to KCs in a second application. After 33 s (the time interval of differentiation between OA and saline induced Ca^{2+} transients, see Figure 1 *C*), 100 µM ACh was applied two times to KCs during a time interval of 45 s. In *C*, the first ACh application induces maximal peaks of Ca^{2+} signals which do not significantly differ between the two experimental groups (Wilcoxon rank sum test: p = 0.069; A1 (1) ACh N = 29; A2 (1) ACh N = 29; the first application is depicted as a red box in A1 and A2). In *D*, box-plots of the normalized Ca^{2+} signals for A1 and A2 are color coded according to the application protocol used. The Ca^{2+} signals are normalized to the maximum peak of the first Ca^{2+} signal induced by ACh. Ca^{2+} signals decrease in a highly significant way after 1 µM OA has been applied compared to the signals of the control group without OA

(Wilcoxon rank sum test: both groups $p < 0.001$). In the experimental group, after OA application, the Ca^{2+} signals decrease significantly during the third and fourth applications of ACh (shown as green and purple in the figure; A2 (3) ACh compared to A2 (4) ACh: $p = 0.001$). In comparison, the control group signals (without OA application) are not significantly different (A1 (3) ACh compared to A2 (4): $p = 0.106$; box-plot structure: a red line indicates the median, a box indicates the first and third quartiles, whiskers indicate the 10^{th} and 90^{th} quartiles, the outliers are above the whiskers).

In patch-clamp recordings, three consecutive ACh applications (100 µM for 400 ms) with time intervals of 135 s decreased the peak currents induced by ACh after OA application (1 µM; bath applied for 135 s; Figure 5 A). Between the first ACh application and the second, OA/serotonin or forskolin were washed in via bath; after the second ACh application, the applied transmitter was washed out for 135 s and again ACh-induced currents were measured (ACh post). All the data were adjusted to compensate for the run-down effects of repetitive ACh-induced inward currents (in KCs the maximum of current decrease for 1% after 45 s; in antennal lobe neurons no run-down effects during measurements of 270 s are observed). Figure 5 *B-E* shows ACh-induced peak currents relative to the first peak current. Inward currents were faster and decreased more strongly after a 1-µM OA application in KCs (mean of ACh+OA: 0.68, SEM: 0.12; mean of ACh post: 0.72, SEM: 0.05; Figure 5 B) than in antennal lobe neurons (mean of ACh+OA: 0.82, SEM: 0.08; mean of ACh post: 0.81, SEM: 0.08; Figure 5 *C*). In addition, 270 s after the first ACh application, the third ACh-induced inward currents are significantly decreased. Furthermore, in antennal lobe neurons, the ACh-induced inward currents were reduced after serotonin was applied (1 µM, applied via bath for 135 s). The adenylyl cyclase activator forskolin (10 µM, applied via bath 135 s) also has the effect of reducing the ACh-induced current in antennal lobe neurons (Figure 5 *E*). The modulatory effects on peak currents after serotonin or forskolin applications seem to slightly recover after a 135 s wash. This recovery was smaller in ACh-induced peak currents after the wash of OA in antennal lobe neurons as well as in KCs. Finally, these findings verify the modulatory effects of OA on ACh-induced Ca^{2+} signals in KCs during Ca^{2+} imaging recordings.

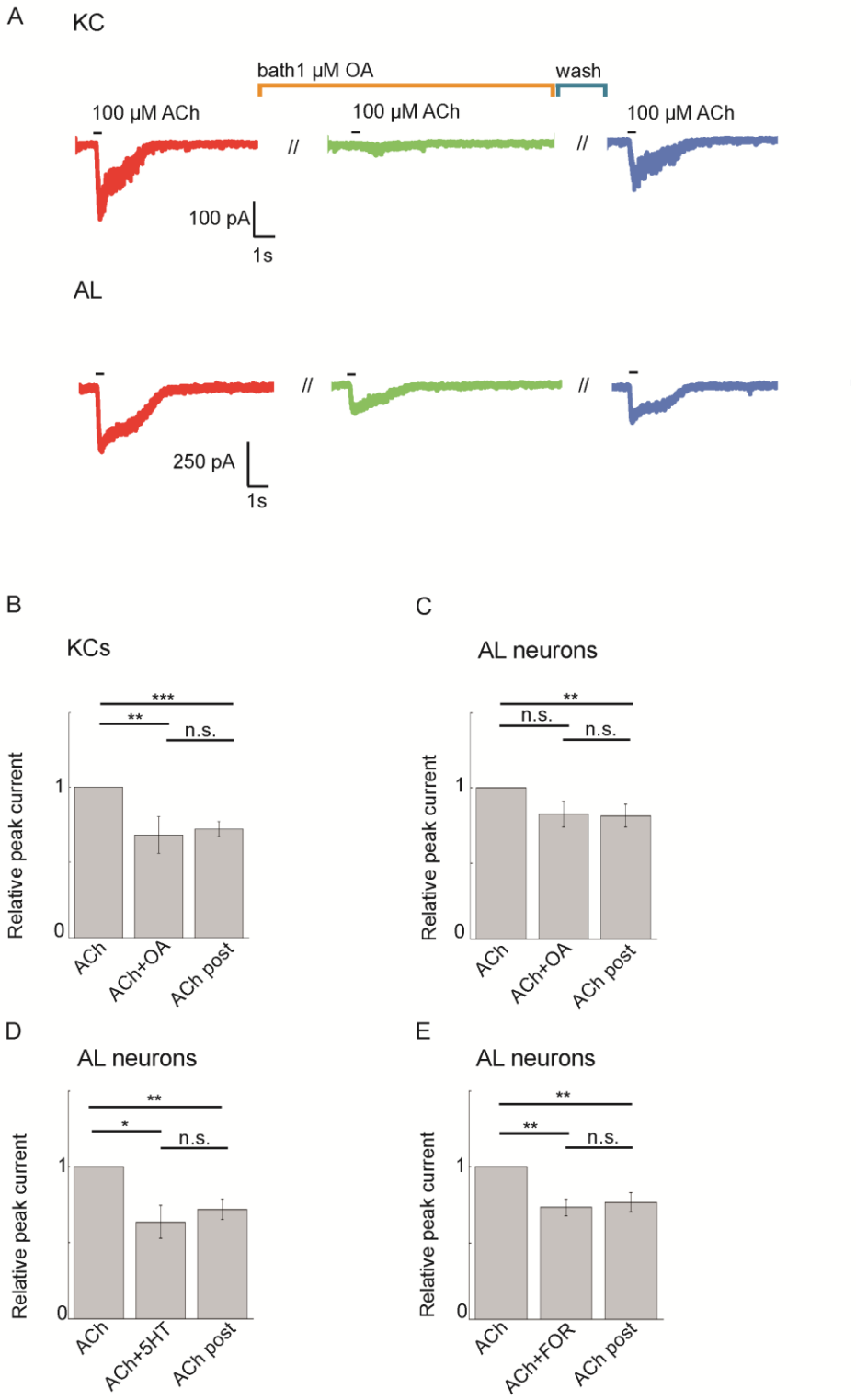

Figure 5 | Modulatory effects of OA and serotonin on ACh-induced inward currents. *A* Representative ACh-induced inward currents (100 μM ACh, for 400 ms, indicated by the small black bar above the currents) are shown in red, and

representative decreased currents after 1-µM OA application are shown in green and blue. The first panel shows currents in KCs, the second in antennal lobe neurons (AL). The OA (5HT or forskolin) was bath applied. The ACh was applied in 135-s intervals. *B* shows significant differences in the relative peak currents after an application of 1-µM OA in KCs (Wilcoxon rank sum test between ACh and ACh+OA: $p = 0.006$; ACh and ACh post: $p < 0.001$; ACh+OA and ACh post: n.s.; $N = 8$). *C* shows that in antennal lobe neurons, the ACh-induced inward currents were less reduced than in KCs, but still significant in the second ACh-induced currents after the OA application (Wilcoxon rank sum test: between ACh and ACh+OA: n.s.; ACh and ACh post: $p < 0.014$; ACh+OA and ACh post: n.s.; $N = 13$). *D* shows significant differences in the relative peak currents after the application of 1 µM serotonin in antennal lobe neurons (Wilcoxon rank sum test: between ACh and ACh+5HT: $p = 0.048$; ACh and ACh post: $p = 0.002$; ACh+5HT and ACh post: n.s.; $N = 6$). *E* shows that forskolin application (10 µM) also results in significantly decreased relative peak currents in antennal lobe neurons (Wilcoxon rank sum test: between ACh and ACh+FOR: $p = 0.008$; ACh and ACH post: $p = 0.008$; ACh+FOR and ACh post: n.s.; $N = 5$).

3.5 Discussion

OA- and serotonin-induced Ca^{2+} signals in KCs and antennal lobe neurons

Experiments with the OA-receptor antagonists mianserin and epinastin demonstrated that the OA-induced Ca^{2+} signals were blocked after OA applications. Interestingly, the serotonin-induced Ca^{2+} signals were also blocked by mianserin and epinastin. OA and serotonin lead to Ca^{2+} response patterns which differ in KCs compared to antennal lobe neurons. The decay of Ca^{2+} signals during a response differs according to cell type. The Ca^{2+} signals in the KCs decay more slowly after serotonin applications than after OA applications. OA has a modulatory effect on the ACh-induced Ca^{2+} signal as well as on the ACh-induced inward currents.

The dynamics of the produced Ca^{2+} rises after OA or serotonin application and varies among cell classes. KCs respond to OA and serotonin in a more oscillatory manner than antennal lobe neurons do. They produce Ca^{2+} transients which are similar to saturation curves. The activation of heterologously expressed AmOA1 receptors in HEK cells expresses similar oscillating signals (Grohmann 2003). How could differences in Ca^{2+} signal patterns be explained in KCs and antennal lobe neurons? Generally, Ca^{2+} dynamics are processed by many different buffering and releasing

mechanisms. Besides compensation by endogenous Ca^{2+} buffers, large increases in intracellular Ca^{2+} are compensated by active extrusion out of the cytosol. Here, one buffering method is the triggering of intracellular Ca^{2+} uptake by mitochondria, which regulate the uptake of intracellular Ca^{2+} into endoplasmic stores by self-releasing Ca^{2+} at moderate levels, thus avoiding destructive intracellular Ca^{2+} rises (review Babcock and Hille 1998). Another Ca^{2+} buffer mechanism is driven by membrane pumps. An increase in intracellular Ca^{2+} is caused by Ca^{2+} influxes through voltage-gated and/or Ca^{2+}-permeable ligand-gated ion channels as well as conducted by the release of Ca^{2+} from the endoplasmic reticulum or mitochondria. The dynamics of the oscillation cycles and other signal patterns depend on these mechanisms and could vary depending on the different cell types according to their cellular requirements and may include different expressed receptor levels and different activated intracellular pathways, networks, and structures.

In our study, the differences in Ca^{2+} signal expression in KCs compared to in antennal lobe neurons as well as differences in decay time indicate these cellular occurrences. The study of Messutat et al. (2001) shows a loss in mitochondrial functions by blocking the Ca^{2+} uptake and releasing stored Ca^{2+} with the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) causes a sustained rise in Ca^{2+} signals in efferent dorsal unpaired median (DUM) neurons of *Periplaneta americana*. These signals are somewhat similar to the expressed Ca^{2+} transients in the antennal lobe neurons in our study. A possible different mitochondrial function in antennal lobe neurons as compared to in KCs may cause different Ca^{2+}-signal patterns. An indicator of different induced pathways and timings as well as different cellular prerequisites may be explain the differences in the half-life of Ca^{2+} decays after OA applications between antennal lobe neurons and KCs. The different pathways and timings of activated OA or serotonin receptors could also lead to significant differences in Ca^{2+} patterns or decay durations. The heterologously expressed *Drosophila* OA receptor, DmOctα1Rb, is desensitized by phosphorylation of a single amino acid residue in the third intracellular loop of the G-protein coupled receptor by PKC and is resensitized by dephosphorylation. This property is assumed to induce oscillations (Hoff et al. 2011). Further, it seems that KCs in our study express small spontaneous Ca^{2+} signals (control group Figure 1 *C*) similar to the signals reported in a study of *Drosophila* KCs (Jiang et al. 2005). This spontaneous activity could lead to

the observed small Ca^{2+} rises over the time, which also could be an underlying factor of the expressed Ca^{2+} responses after OA and serotonin applications. These small rises might cause the irreversibility of OA- and serotonin-induced Ca^{2+} responses over the time (Figures 4 and 5).

No selective antagonists of the OA receptor

The well-known antagonists of the OA receptor, mianserin and epinastin, blocked the OA-induced Ca^{2+} signals in cultured KCs. But surprisingly, serotonin-induced Ca^{2+} responses were also blocked antagonistically, leading to the assumption that both antagonists also have an impact on the serotonin receptor. In the past, many behavioral and pharmacological studies included these antagonists to selectively act on the OA receptor of honeybees and also on OA receptors of many other insect species (regarding only during learning and memory formation: in honeybees: Farooqui et al. 2003, 2007; in crickets: Mizunami et al. 2009; in flies: Burke et al. 2012). The inhibitory impact on the serotonin receptor should be investigated in future pharmacological studies. In recent studies, epinastin, a vertebrate H1 histamine receptor antagonist, has been reported to also inhibit the honeybee dopamine receptor (AmDOP2; Beggs et al. 2011). The same study also revealed that mianserin, the vertebrate selective antagonist of the 5HT2 receptor, acts antagonistically on the AmOA1 and AmDOP2 receptors. So far, no other selective antagonists for the OA receptors of the honeybee have been reported. In light of our results, more investigation of these antagonists still seems necessary, especially in regard to all arguments for the honeybee as a versatile model organism for studying learning and memory, particularly as a model organism in which the octopaminergic modulatory neurons take the essential role in mediating the reward during classical conditioning.

Modulations of ACh-induced Ca^{2+} signals and inward currents

OA has a modulatory effect on ACh-induced Ca^{2+} signals in KCs and on inward currents in KCs and antennal lobe neurons. Similar effects are apparent after serotonin applications in antennal lobe neurons. Therefore, we could show a

modulatory interaction by activating an essential receptor of the reward with OA and by activating the nAChR, representing the olfactory pathway during olfactory conditioning.

The application protocol during modulation of Ca^{2+} imaging experiments followed the timing of the observed Ca^{2+} signals after OA stimulations in KCs. Here, after 33 s the observed Ca^{2+} responses to OA were significantly differentiated from those of the control group. Therefore, ACh and OA were not simultaneously applied in the modulation protocols; instead, we applied OA 33 s before ACh. Hence, the significantly stronger Ca^{2+} signals found in the test group compared to the control group suggest that the responses to OA and to ACh are concurrently expressed, even though both receptors were activated at different times. Following this line of reasoning, one could also maintain the idea of coincident activation of two pathways in the presented preparation. Regarding our *in vitro* experiments with the primary neuron culture, the activation timings may be different and may therefore not represent the timing during classical conditioning *in vivo*. But our preparation may still shed light on possible receptor interaction and modulation during the coincident activation of both essential olfactory learning pathways.

What are the cellular mechanisms of decreased ACh-induced Ca^{2+} signals and inward currents after OA and serotonin receptor activation? The observed decreased Ca^{2+} signals and inward currents are the results of intracellular modulatory changes. After additionally applied OA/serotonin along with ACh, the activation of the two possible pathways can be assumed to induce the following cellular mechanisms, as shown in the hypothetical model in Figure 6: 1. Through the activation of the nAChR and a G_q-protein coupled receptor, which induces a Ca^{2+} signal via cytosolic IP_3 release, an elevation of intracellular Ca^{2+} could activate an as yet unknown Ca^{2+}-dependent kinase. A Ca^{2+}-dependent PKC is one possible kinase which could be activated. The PKC is believed to play an important role during synaptic plasticity and memory formation by phosphorylating target proteins and is reported to be activated by the CS as well as by the US in antennal lobe neurons (Grünbaum and Müller 1998). But so far its specific role during consolidation and memory formation is still unknown. In *Drosophila*, PKC is reported to phosphorylate the odorant receptor and regulate its function (Sargsyan et al. 2011). A similar function may be assumed by Ca^{2+}-dependent kinases by phosphorylating the intracellular part of the nAChR,

leading to changes in the receptor conductance in KCs and in antennal lobe neurons of the honeybee. 2. The decreased inward currents found in the antennal lobe neurons after the application of the adenylyl cyclase activator, forskolin, suggests the existence of another possible pathway: the activation of cAMP/PKA signaling. This could be via an adenylyl cyclase coupled to an unidentified β-adrenergic-like OA receptor or via a similar adenylyl cyclase activation through the 5HT7 receptor. Also, a cAMP/PKA pathway could be activated through a Ca^{2+}/calmodulin-dependent AmAC8 or another unknown adenylyl cyclase. A cAMP/PKA signaling pathway may also lead to a phosphorylation via the PKA of the nAChR (Huganir and Greengard 1990). In crickets' KCs, the voltage-sensitive Ca^{2+} channel (VSCC) is the target of PKA phosphorylation, which leads to decreased Ca^{2+} responses after OA-induced cAMP/PKA signaling pathway (Kosakai et al. 2008). In our preparation during the Ca^{2+} imaging experiments, this mechanism may not be completely excluded as insignificant compared to the modulatory changes of the nAChR during patch-clamp recordings. During these experiments, with a holding potential of -70 mV, the activation of VGCC can be excluded. Hence, we primarily assume a modulatory effect of OA/serotonin through a Ca^{2+}-activated intracellular pathway which ends in phosphorylation of the nAChR, caused by a Ca^{2+} dependent unknown kinases or the PKA.

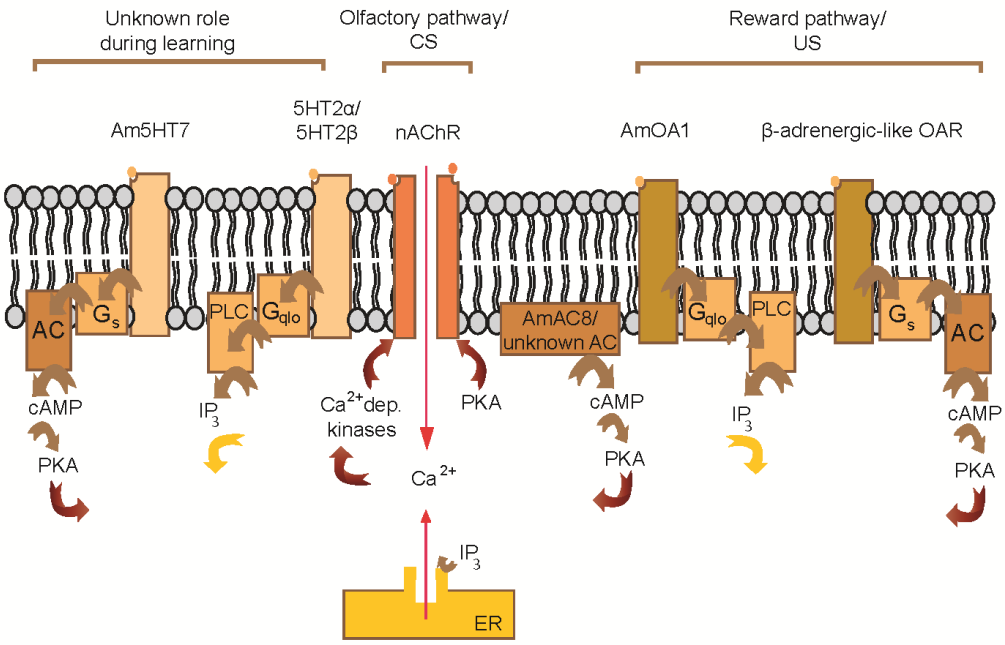

Figure 6 | Model of the cellular mechanisms underlying olfactory learning. The schematic diagram depicts the cellular physiology in KCs and antennal lobe neurons where the unconditioned stimulus (US) and conditioned stimulus (CS) pathways could converge. The olfactory pathway activates the nAChR, leading to a strong Ca^{2+} and sodium influx and therefore depolarizes the postsynaptic membrane in antennal lobe neurons and KCs. The reward could activate either the AmOA1 receptor, which induces a Ca^{2+} efflux out of the endoplasmic reticulum (ER) by mediating intracellular inositol triphosphate (IP_3) release. Or the reward could activate an identified but not yet characterized β-adrenergic-like OA receptor, which mediates a cAMP/PKA signal through a receptor positively coupled to an adenylyl cyclase (indicated as AC). Both pathways could have modulatory effects on ACh-induced inward currents: 1. By activating the AmOA1 receptor, an elevated intracellular Ca^{2+} increase may activate Ca^{2+}-dependent kinases, which in turn could phosphorylate the nAChR. 2. By activating a cAMP/PKA signal through β-adrenergic-like OA receptor activation, thereby allowing the PKA to phosphorylate the nAChR. Further, an unknown adenylyl cyclase or the AmAC8 could mediate the cAMP/PKA pathway. Therefore, both phosphorylation agents, the Ca^{2+}-dependent kinase and PKA, could lead to modulatory conductance changes in the nACh and may therefore act as coincidence detectors of the convergence of the CS and US during conditioning. The role of serotonin receptors during learning are still unknown. But the indicated serotonin receptors (5HT2α/5HT2β and Am5HT7) may induce intracellular pathways similar to

those of the two OA receptors. (Brown arrows indicate the activity-dependency and yellow arrows stand for the IP_3 signaling pathway. Red arrows indicate the possible directly or indirectly modulatory effect on the nAChR.)

In *Drosophila*, the main accepted cellular mechanism for olfactory associative learning includes the activation of the cAMP/PKA pathway, activated by the *rut* AC. The *rut* AC has been shown to be activated by an additive elevation of $[Ca^{2+}]_i$ caused by the Ca^{2+} influx through activation of the nAChR and by the activation of a G-protein coupled receptor (Tomchik and Davis 2009). Therefore, the *rut* AC acts as a molecular site of convergence between the CS and US which induces learning-dependent plasticity through the activation of the cAMP/PKA pathway. The study of the dopamine modulations of *in vivo* ACh responses in the *Drosophila* mushroom body adds the idea of the common supposition of a single cAMP signaling mechanism during olfactory learning. The study attributes a central role to $[Ca^{2+}]_i$ signals in this form of learning (Tsydzik and Wright 2009). The authors show Ca^{2+} signals after ACh or dopamine which were iontophoretically applied to the mushroom body. They assumed that the activation of another dopamine receptor like the DmDOP1 in addition to the DopR99B leads to an increase in $[Ca^{2+}]_i$. The simultaneous application of ACh and dopamine reduces the Ca^{2+} signals in mushroom bodies. These results could be related to our conclusion that the modulatory changes of the nAChR occur via a Ca^{2+} signaling pathway.

Regarding the role of KCs and antennal lobe neurons during learning and memory formation, the question is what the functional significance of decreased ACh-induced Ca^{2+} signal or inward currents after OA applications could be. The mushroom body extrinsic output neurons induce changes in the neuronal activity during olfactory reward learning (Mauelshagen 1993; Rybak and Menzel 1998; Grünewald 1999; Okada et al. 2007; Haehnel and Menzel 2010). Maulshagen found in pioneering intracellular recordings of the extrinsic neuron PE1 that the activity decreased during the first conditioning trails compared to bees, which were only stimulated with an odor. These findings may be related to our findings of OA-induced decreasing ACh responses in KCs. These may lead to decreased output signals to downstream effected extrinsic neurons, such as the PE1. Additionally, the findings of Grünewald (1999) regarding intracellularly recorded mushroom body feedback neurons revealed

that shortly after the stimulus pairing of olfactory conditioning, odor-induced spike activity decreased. Consequently, KCs may provide inhibitory output to feedback neurons, which may be caused by possible decreased input at the KC postsynaptic site. During odor-induced activity *in vivo* measurements of KCs, the KCs themselves showed sparse responses (Szyszka et al. 2005), whereas the KC responses became long lasting (Szyszka et al. 2008) and stronger (Faber and Menzel 2001) upon odor–sucrose pairing during conditioning. These dynamics in activity *in vivo* might be provoked by the neuronal network of the mushroom body neuropil instead of the plasticity changes in a single cell as described in our findings. Similar network-dependent activity changes were studied in the antennal lobe *in vivo* (Rein et al. 2013). The Ca^{2+} responses after OA and odor presentation are modulated in the antennal lobe, not uniformly, which is assumed to depend on the neuronal network within the antennal lobes. Rein et al. (2013) hypothesize that OA modulations are provoked only by local interneurons which converge onto projection neurons within the antennal lobes. But evidence of the unique expression of AmOA1 in LNs within the antennal lobe is missing so far (Sinakevitch et al. 2011). Due to our finding of the consistent modulatory effects of OA as well as of serotonin on the ACh-induced inward current in antennal lobe neurons, we postulate that in the primary neuron culture of the antennal lobes, different cell types probably express the AmOA1 receptor on the same level. These observations suggest that antennal lobe neurons as well as KCs have similar cellular requirements, such as receptor expression and intracellular pathways that lead to the observed modulatory effects via OA within the primary neuron culture. Furthermore, our preparation allows us to study the direct modulatory effects of the OA on ACh-induced inward currents and Ca^{2+} responses. On the single cell level, this preparation leads to a reliable model system for studying modulatory interactions and the modulations of receptors and could provide ideas how neuronal networks could compute the convergence of the CS and US in the lively honeybee.

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CHAPTER 4[3]

4 *In vitro* CREB stimulations of honeybee Kenyon cells

4.1 Abstract

Studies in several vertebrate and invertebrate model organisms have shown that the cAMP/PKA-dependent phosphorylation of the cAMP-response element-binding protein (CREB) induces learning-related cellular plasticity by mediating the transcription after behavioral learning. In honeybee neurons, it is assumed that a G_S-protein-coupled OA receptor is expressed in KCs as well as in antennal lobe neurons, possibly a β-adrenergic-like octopamine (OA) receptor which induce a cAMP/PKA pathway and a CREB signaling. Two serotonin receptors, the 5HT1A receptor and 5HT7 receptor, are molecularly and functionally described which activate or deactivate a cAMP/PKA pathway and additionally may induce phosphorylation or dephosphorylation of CREB. In our study, we address the question of whether the activation of these G_S-protein-coupled receptors would lead to the phosphorylation or dephosphorylation of CREB. We tested this in the primary neuron culture of Kenyon cells (KCs) of the mushroom body neuropil, where learning-related plasticity occurs. We studied the time dependencies of the phosphorylated CREB after stimulation with OA and serotonin. We found a distinct amount of phosphorylated CREB in all the control groups incubated with pure saline. The experimental groups stimulated with OA and the adenylyl cyclase activator forskolin were not significantly different from the controls. Surprisingly, after serotonin stimulations lasting 0.5 min, the level of the phosphorylated CREB decreased compared to the level in the controls.

[3]This chapter is based on the following manuscript: "*In vitro* CREB stimulations of honeybee Kenyon cells" by Himmelreich S, Gampe T, Grünewald B

Experiments and data analysis

Sophie Ziegler-Himmelreich designed the experiments. Sophie performed the experiments with Kristine Gampe. Kristine introduced Sophie into immunocytochemistry and Sophie could use Kristine´s laboratory place. All data analysis was done by Sophie Ziegler-Himmelreich.

Writing process and figure designing

Sophie Ziegler-Himmelreich wrote the manuscript and designed and developed all figures. Bernd Grünewald provided suggestions and criticisms on parts of the text.

4.2 Introduction

CREB is phosphorylated by PKA and induces the expression of genes that play a crucial role in the formation of long-term synaptic plasticity in various species. Studies in invertebrates like *Aplysia* and *Drosophila* showed that the phosphorylation of CREB is a prerequisite of consolidation and long-term memory (Brunelli et al. 1976, Yin et al. 1995, for review, Kandel 2012).

In the honeybee, the exact pathway which induces the phosphorylation of CREB has not yet been determined, although several studies of the cAMP/PKA pathway within the antennal lobe neurons or the intrinsic neurons of the mushroom bodies, the KCs, have revealed that this pathway is essential for memory formation (reviewed by Eisenhardt 2006). Therefore, this pathway is hypothesized to induce phosphorylation of CREB during learning and memory formation. Several studies of different learning paradigms, such as olfactory conditioning or the habituation of the proboscis extension reflex, attempted to unravel the intracellular pathways mediating learning-related cellular plasticity: (1) PKA is involved in the formation of early long-term memory (LTM) and late LTM, which last for 1-2 days and 3-4 days, respectively (Müller 2000). (2) After habituation of the proboscis extension reflex, PKA is activated (Müller and Hildebrandt 2002). (3) Stimulation with OA, the reward transmitter during olfactory conditioning, increases the PKA activity in antennal lobe neurons (Hildebrandt and Müller 1995). (4) The down-regulation of the gene expression of the catalytic subunit by antisense oligionucleotide injections during and shortly after olfactory conditioning leads to a slight impairment of LTM retention (Fiala et al. 1999).

How could the cAMP/PKA pathway and therefore the phosphorylation of CREB be activated in honeybee neurons? In *Drosophila*, the rutabaga adenylyl cyclase (*rut* AC) is similar to the mammalian type 1 adenylyl cyclase. They are both stimulated by G_s-coupled receptor activation and the intracellular elevation of Ca^{2+}/calmodolin (Livingstone et al. 1984, Levin et al. 1992). Tomchik and Davis 2009 found that *Drosophila* mushroom body neurons showed cAMP responses to either depolarization (via acetylcholine stimulation) or dopamine applied in isolation in wild-type and rutabaga mutant flies. This indicates an activation of adenylyl cyclases which respond to unpaired dopamine and acetylcholine (ACh) stimulations. Notably, only the *rut* AC showed synergistic increases in cAMP upon coincident depolarization

through ACh and dopamine stimulation, with the latter as the transmitter of the aversive stimulus. Interestingly, the opposite effect was observed after stimulation with OA, which is released during appetitive conditioning in *Drosophila*. In honeybees, ACh stimulations or the elevation of intracellular Ca^{2+} through the Ca^{2+}-ionophore A21187 or KCl applications could not be shown to lead to PKA activation (Müller 1997), although the Ca^{2+}/calmodulin-dependent AmAC8 was found in honeybee mushroom bodies in recent studies (Balfanz et al. 2012). Only micromolar concentrations of OA significantly increases the PKA activity, indicating an β-adrenergic-like OA receptor coupled to an as yet unknown AC (Müller 1997). Besides the OA receptor, serotonin (5-hydroxytryptamine, 5HT) also activates through the 5HT7 receptor a cAMP/PKA pathway and is reported to be expressed in intrinsic mushroom body neurons (Schlenstedt et al. 2006). In addition, the 5HT1A receptor was found in mushroom body intrinsic neurons as well. Activation of this receptor, however, leads to an inhibition of cAMP synthesis (Thamm et al. 2010). Less is understood about the entire activation of the pathway from the activated receptor to the intracellular cAMP/PKA signaling, ending in the phosphorylation or dephosphorylation of CREB.

In our study, we tested the effect of OA and serotonin stimulation on the level of phosphorylated CREB in cultured KCs. The findings show that KCs have a distinct level of phosphorylated CREB. With the different stimulation schedules of 0.5 min, 10 min, and 60 min, the time-dependent phosphorylation levels of CREB were significantly different after serotonin stimulations. Serotonin has a decreasing effect on the phosphorylation level of CREB during stimulation of 0.5 min, which could indicate a possible serotonin-inducing inhibitory pathway in our preparation. However, the OA stimulations showed only tendencies in the 10-min group. Here the phosphorylated CREB level was slightly increased compared to that of the control group.

4.3 Materials and methods

The Kenyon cells of the honeybee, *Apis mellifera carnica*, of the pupal stages P6-7 were dissected following a protocol modified from the protocol of Kreissl and Bicker (1992). After the dissection of the mushroom bodies, the neuropil was first transferred

into a calcium-free solution (mM: 130 NaCl, 5 KCl, 10 $MgCl_2$, 25 glucose, 180 sucrose, and 10 Hepes; pH 7.2) for cell separation and then into preparation medium (Leibovitz L15 medium, Gibco BRL, supplemented with 22.2 mM sucrose, 22.2 mM fructose, 0.09 mM glucose, 0.029 mM proline M, 0.05 ml/l penicillin/streptomycin, and 5 µl/l gentamycin; 500 ± 10 mOsmol l^{-1}). Gentle trituration with a pipette dissociated the cells completely. The cells were allowed to settle and adhere to sterile concavalin A-coated round glass cover slips with a diameter of 10 mm (Sigma-Aldrich, Germany). The cover slips were then placed in a 24-well plate. For each cover slip, we dissected five mushroom bodies to yield a constant cell distribution. After 45 min, the cells were floated with culture medium (containing Leibovitz L15 medium, see protocol above, and supplemented with 2 mM Pipes, 14.9% FCS, and 1.2% yeastolate) and were kept by 26 °C for 24 h in an incubator.

Immunocytochemical analysis of phosphorylated CREB

Approximately 2 h before cell stimulation, the culture medium was replaced by standard saline (in mM: 130 NaCl, 6 KCl, 4 $MgCl_2$, 5 $CaCl_2$, 10 Hepes, 25 glucose, 170 sucrose, 500 ± 10 mOsmol l^{-1}, pH 6.7). For CREB stimulation, the cells were incubated for 0 min, 0.5 min, 10 min, and 60 min with 1 µM OA, 1 µM serotonin, or 10 µM forskolin, all dissolved in standard saline. Pure standard saline served as a control. The fixation and immunocytochemical labeling was performed as described in Grimm et al. 2009: After CREB stimulation, the cells were fixed on ice for 20 min with 2% paraformaldehyde in phosphate-buffered saline (PBS; in mM: 137 NaCl, 3 KCl, 15 Na/K, phosphate buffer, pH 7.4) containing a mixture of phosphatase substrates, proteases, and phosphatase inhibitors as follows (in mM): 25 NaCl, 10 NaF, 10 Na4P2O7, 25 sodium-β-glycerophosphate, 25 p-nitrophenyl phosphate, 0.5 EGTA, 1 PMSF, 1 Na3VO4, 0.1 okadaic acid, 10 µg/ml leupeptin, 10 µg/ml antipain. Subsequently, the cells were washed twice with washing buffer (PBS including phosphatase inhibitors, as mentioned above), permeabilized (0.1% TritonX100 in washing buffer) for 30 min, and incubated with blocking solution (5% bovine serum albumin in washing buffer) for another 60 min on ice. The cells were incubated over night at 4 °C with an antibody against the phosphorylated form of CREB (1:100, # 9198, Cell-Signaling, USA) diluted in 1% BSA in washing buffer. The

cells were washed the next day with PBS. The incubation of the second antibody (1:400, IgG/Cy3-conjugated, Dianova, Germany) together with the fluorescent dye DAPI (Diamidino-phenylindole,1 µg/ml, the blue-fluorescent DAPI nucleic acid stain preferentially stains dsDNA and is therefore an indicator for the nucleus of a cell, Sigma-Aldrich, Germany) was performed for 1 h at room temperature in a dark chamber. After three washing steps with PBS, the cells were embedded in Aqua-Poly/Mount (Polyscience Europe GmbH, Germany).

Fluorescence microscopy and data analysis

Images were taken with a charge-coupled device camera (CCD camera, AxioCAM MRm, Zeiss, Oberkochen/Jena, Germany) mounted on an inverse fluorescence microscope (Axiovert 200, Zeiss). The images have 1382 x 1030 pixel resolution in the x-y plane and an intensity resolution of 150 pixels/inch. Depending on the cell density, 3 to 10 pictures were taken of each cell dish to obtain a minimum of 500 recorded cells. The analysis of the phosphorylated CREB level was conducted as follows: 1. The cells which showed a double staining of DAPI and phosphorylated CREB were counted. 2. The KCs showed a distinct level of phosphorylated CREB; to compare the phosphorylated CREB levels among the experimental groups, the reference value for normalization was determined by the maximum brightness in each image, yielding normalized values for light-intensities in each image. 3. Weak stainings of phosphorylated CREB were not counted (beneath a determined threshold of light intensity of 30% related to the maximum in each image, see Figure 1D) 4. The normalized light-intensities of phosphorylated CREB were related to all the counted DAPI-phosphorylated-CREB positive neurons in each image (in the following indicated as percentage of phosphorylated CREB level). The resulting phosphorylated CREB level in groups stimulated during 0 min, 0.5 min, 10 min and 60 min was then divided by the median of the phosphorylated CREB level of the respective control group, yielding the ratio between stimulated group and control. The analyses and statistical calculations were done via a custom-written analysis routine in MATLAB (Version 2012a, The MathWorks, Inc., Natick, MA, USA).

4.4 Results

Decreasing phosphorylated CREB level after serotonin stimulations

We find that KCs have a distinct level of phosphorylated CREB in all tested groups. We counted only the cells with a double staining of DAPI/phosphorylated CREB and the staining of phosphorylated CREB had to be strong (bright) enough to be counted (Figure 1).

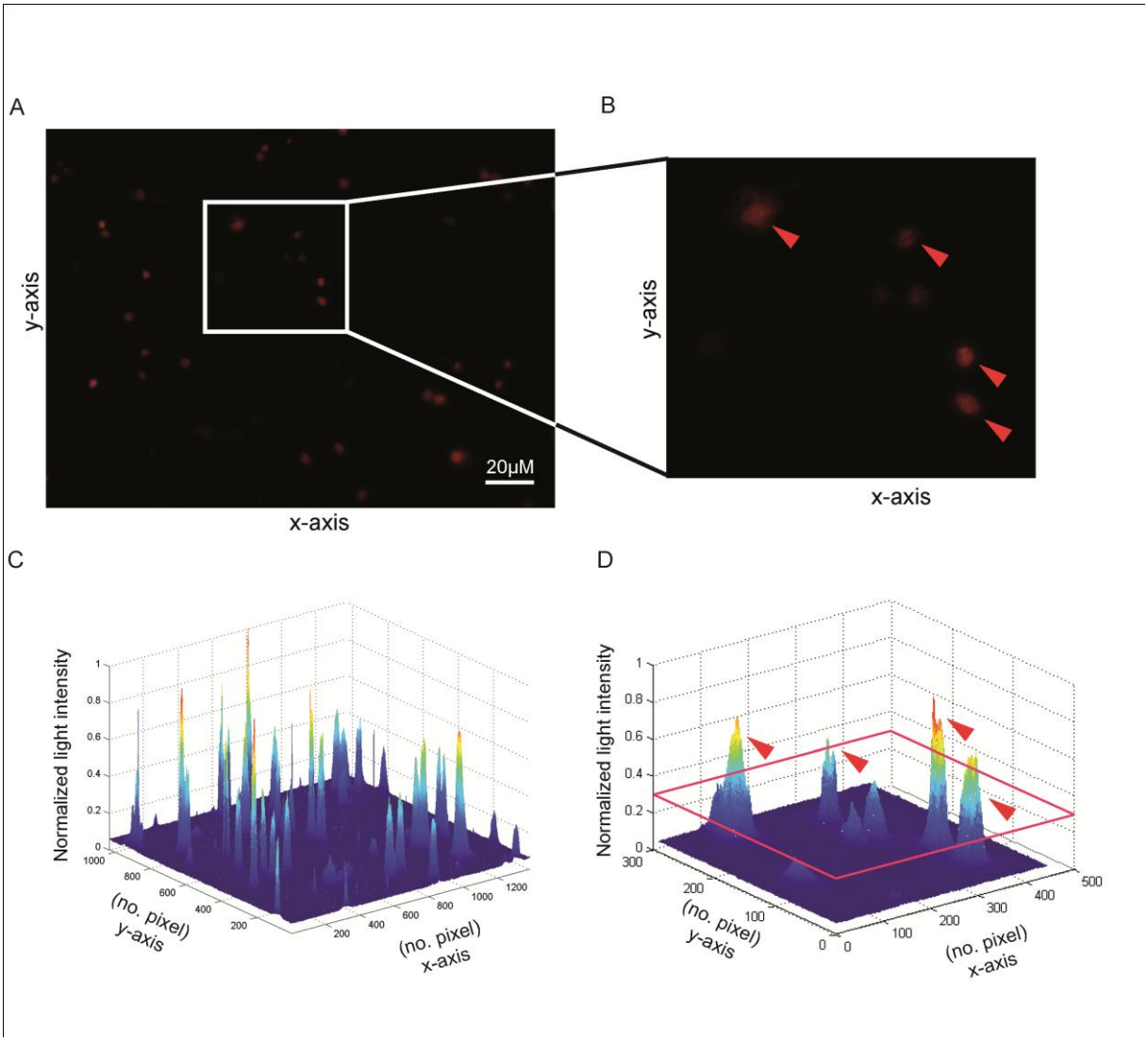

Figure 1 | *In vitro* CREB stimulations of 1 µM serotonin after 0.5 min in KCs. *A* Representative staining of phosphorylated CREB after serotonin stimulation during 0.5 min (phosphorylated-CREB positive cells are red). *B* Magnification of a part of the image in *A*. The red arrows indicate the cells which are counted as phosphorylated-CREB positive. *C* and *D* show the normalized 3D plot of the image in *A* and *B*, respectively. *C*: Light intensities of phosphorylated CREB were normalized to the brightest cell. *D* Magnification of a part of the plot in *C*. Cells were counted as

phosphorylated-CREB positive above the determined threshold indicated by the red square.

The control groups showed a phosphorylated CREB level (medians of the control group for 0 min, 0.5 min, 10 min, and 60 min stimulations as percentages: 11%, 13%, 4%, and 10%, respectively. Figure 2). Hence, we also made measurements in experimental groups which were not stimulated but had the stimulation solution applied together with the fixation solution (0-min stimulation). In these groups, the phosphorylated CREB levels were similar to those levels in the control group with pure saline stimulation (medians of the 0-min stimulation group for OA, serotonin and forskolin as percentages: 8%, 8%, and 12%, respectively. Figure 2). A strong effect was found after 0.5-min stimulations with serotonin (1µM). Here, the phosphorylated CREB level is significantly decreased compared to the phosphorylated CREB level of the control group with saline. The group stimulated with OA only showed tendencies of a stronger effect on phosphorylated CREB compared to the control groups: after 10-min stimulation, the phosphorylated CREB level was slightly increased.

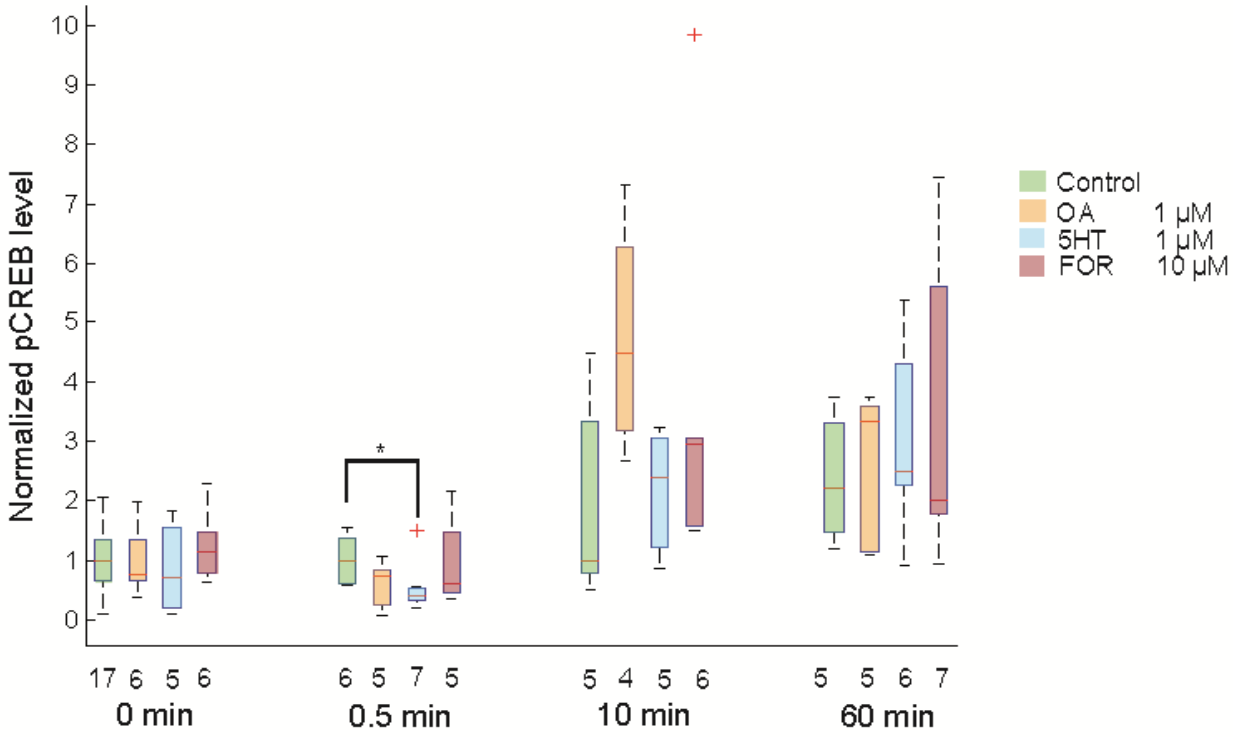

Figure 2 | Phosphorylated CREB after stimulations with OA, serotonin, and forskolin. The results of normalized phosphorylated CREB levels are depicted as box plots. The resulting phosphorylated CREB levels of the experimental groups with stimulants OA, serotonin, and forskolin are normalized to the respective control groups (stimulated with pure saline) using the same stimulation protocol. Significant differences are found between the control and the group with 0.5-min serotonin stimulation (Wilcoxon rank sum test: $p = 0.022$. The sample sizes are indicated as numbers under the box plots. In the box plot: a red line indicates the median, a box indicates the 1st and 3rd quartiles, a whisker indicates the 10th and 90th quartiles and small red an outlier.

4.5 Discussion

For the first time, the phosphorylation of CREB has been stimulated in an *in vitro* approach, most likely activating a possible β-adrenergic-like OA receptor, and two serotonin receptors, 5HT1A and 5HT7, which are all coupled to an adenylyl cyclase. The activation of these receptors results in an activation of an adenylyl cyclase and in a positive or negative inducing cAMP/PKA pathway. With respect to stimulation with OA, serotonin, or with the adenylyl cyclase activator forskolin, we did not find any remarkable changes in phosphorylated CREB level. This could be due to the following: 1. The changes may not be statistically significant due to the small sample size of our study. We found tendencies of increasing effects on the phosphorylated CREB level only after the 10-min OA stimulations. 2. Because one serotonin receptor, 5HT1A, is reported to lead to a decreasing cAMP/PKA signaling pathway and to a possible dephosphorylation of CREB, while the other serotonin receptor, 5HT7, leads to an increasing pathway, the effects of one or the other could either partially or completely cancel each other out within the preparation. This supposition could also explain the great variability within the 10-min group or 60-min group. 3. Forskolin should serve as an adenylyl cyclase activator and therefore as a control for inducing a cAMP/PKA pathway without directly activating a distinct receptor. But forskolin did not induce any remarkable effects. This may occur because forskolin activates any adenylyl cyclases in the neuron, where we do not know how fast they are activated or deactivated due to the artificial character of forskolin. Hence, our preparation appears to be less specific in recording forskolin effects on the phosphorylation CREB level.

Interestingly, stimulating with serotonin led to decreasing levels of phosphorylated CREB in the 0.5-min stimulations. This could indicate a possible activation of the 5HT1A receptor, which is reported to inhibit the signaling of cAMP. This was tested in HEK cells where the 5HT1A receptor was heterologously expressed (Thamm et al. 2010). In that study, a dose-response relationship of serotonin on the intracellular cAMP level ($[cAMP]_i$) was examined with serotonin concentrations ranging from 1 nM to 30 µM. Maximal attenuation of cAMP synthesis was observed at serotonin concentrations of ≥ 3 µM. In our study, the activation of a possible 5HT1A receptor could also lead to a block of cAMP synthesis and therefore to the observed decrease of phosphorylated CREB level. In further studies, it would be interesting to simulate

the receptor activation with agonists of serotonin receptors, such as 5-carboxamido tryptamine and 5-methoxytryptamine. By the use of the putative antagonists of serotonin receptors (methiothepin or prazosin), the effects could be reversed, similar to the findings of the authors Thamm et al. (2010).

An increasing effect of OA on the phosphorylated CREB level was observed after 10-min of stimulation, an effect which was not significant due to the great variability of phosphorylated CREB levels and the small sample size of the experimental group. However, these findings may indicate an OA receptor activation which leads to a phosphorylation of CREB. These findings of a possible relation between the OA receptor activation and the increasing phosphorylation of CREB would perfectly fit cellular learning models. OA, as the reward transmitter, induces learning related cellular changes through the activation of transcription factors, leading to possible synaptic changes in neurons where learning and memory formation takes place.

4.6 References

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