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Cellular physiology of olfactory learning in the honeybee brain

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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. 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 Ca^{2+} -dependent kinases. (2) Activation of a cAMP-dependent PKA may lead to phosphorylation of the nAChR and hence to learning-related intracellular changes.

Apis mellifera / coincidence detection / acetylcholine receptor / octopamine receptor / antennal lobe / mushroom body

1. INTRODUCTION

Learning processes may 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, *Apis mellifera*, 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; Blenau et al. 2011, this issue). Honeybees can be conditioned to extend their probosces (proboscis extension response) 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 (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

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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 and Müller 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 and Menzel 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 suboesophageal ganglion, the VUM_{mx1} (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 octopamine receptor (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.

2. 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.

2.1. 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 and Alison 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 4,000) of LNs is putatively GABAergic. Consequently, blocking inhibitory synaptic transmission affects odor discrimination and odor-induced spatio-temporal activity (Stopfer et al. 1997; Sachse and Galizia 2002; Deisig et al. 2010).

PNs transmit olfactory information from the AL to the lateral protocerebral lobes (IPL) 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 and Bicker 1989). The PNs are differentiated into lateral PNs (IPNs) 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 with the phasic-tonic responses of the IPNs (Krofczik et al. 2009; Deisig et al.

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: VUM _{mx1}	In vivo intracellular recordings	VUM _{mx1} neuron mediates the reinforcement in associative learning	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	Fernandez 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; IPN: 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	Szyszkta et al. (2005)
MB: lip region	In vivo Ca ²⁺ imaging	Rewarded odors activate MB KCs more than unrewarded	Faber and Menzel (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	Szyszkta 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 and Manz (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 and Menzel (2010)
MB feedback neurons (A3 cluster)	In vivo intracellular recordings	After odor-reward pairing mainly decreased odor induced activity	Grünewald (1999b)

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—IACT 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 and Rössler 2010).

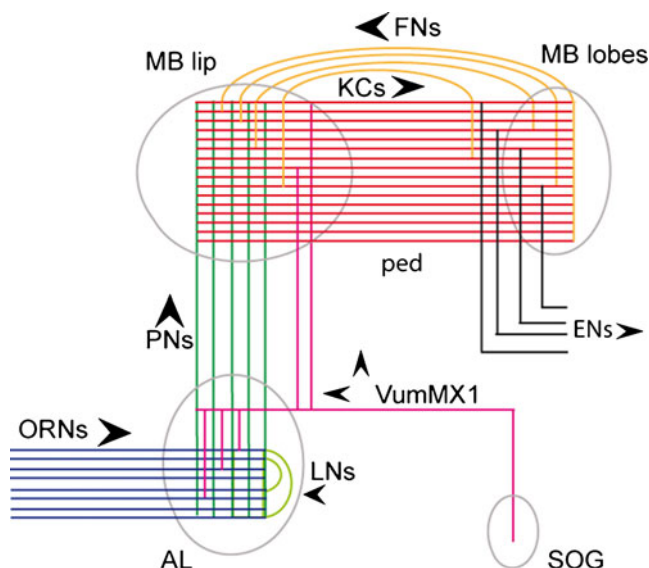


Figure 1. Main neuropils with in- and output regions of the associative olfactory pathway: olfactory input is provided by axons of olfactory receptor neurons (*ORNs*) via the antennal nerve. They converge onto local interneurons (*LN*s) and projection neurons (*PN*s) of the antennal lobes (*AL*). *PN*s leave the *AL* via *ACT*s to the mushroom bodies (*MB*) input region—the lip region of the calyces—where they synapse onto Kenyon cells (*KCs*). Axons of the *MB* intrinsic *KCs* descend via the peduncle (*ped*) to the *MB* lobes. *MB* feedback neurons (*FN*s) 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 suboesophageal ganglion (*SOG*) represents the reward pathway. Branches of this putative octopaminergic neuron synapse onto *AL* neurons and *KCs*.

2.2. 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 and Menzel 1993; Strausfeld 2002). In the *MB*, olfactory information is transmitted to the lip region via the *PN*s. It converges with other sensory information such as visual, gustatory or mechanosensory input (Erber et al. 1987; Gronenberg 2001) and with input from the VUM_{mx1} neuron of the suboesophageal 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 and Menzel 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 and Menzel 1993; Grünewald 1999a, b; Ganeshina and Menzel 2001).

3. 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 amnesic effects (Menzel et al. 1974; Erber et al. 1980). (2) The VUM_{mx1} neuron innervates the antennal lobes and the MB. Spike activity in the VUM_{mx1} 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 and Menzel 1998). (4) Odor-induced activity is modified by learning in AL neurons (Faber et al. 1999), MB extrinsic neurons (Mauelshagen 1993; Rybak and Menzel 1998; Grünewald 1999b; Okada et al. 2007; Haehnel and Menzel 2010) and KCs (Szyszka et al. 2008; Faber and Menzel 2001). (5) Prolonged activation of the PKA within the AL induces behavioral LTM (Müller 2000). (6) Learning modulates protein kinase C (PKC) activity within the ALs and blocking the constitutively active form of the PKC leads to a memory impairment (Grünbaum and Müller 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 and Menzel 2010). The synaptic mechanisms by which MB neurons are modulated are as yet unknown. Menzel and Manz (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 IPNs 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 Ca²⁺ changes and spike activity in AL PNs and extrinsic MB neurons.

In the honeybee, an identified neuron—the VUM_{mx1} 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 VUM_{mx1} neuron paired with an odor is sufficient to substitute for the rewarding properties of the unconditioned stimulus. The VUM_{mx1} 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 VUM_{mx1} neuron is probably octopaminergic (Kreissl et al. 1994; Sinakevitch et al. 2005). The widespread distribution of the OA 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 and Menzel 1998).

4. 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 nicotinic acetylcholine receptor (nAChR), ionotropic GABA receptors (GABAR) and at least two different glutamate receptors.

4.1. Acetylcholine receptors

ACh is the major excitatory transmitter in the nervous system of insects (for review, see Jones and Sattelle 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 and Bicker 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 (for a review, see 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, Amel α 1-9 and Amel β 1-2, the stoichiometry of the insect nAChR has not yet been elucidated (for a review, see Jones and Sattelle 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, 2005): The Amel α 8 (described as Amel α 3 by Thany et al. 2003) is closest to the vertebrate α 3 subunit and is found in pupal KCs and AL neurons (Jones and Sattelle 2010). The Amel α 5 and Amel α 7 (originally classified as Apis α 7-2 and Apis α 7-1, respectively, in Thany et al. 2005) subunits are expressed in both neuropils: MB (KCs type II or clawed KCs) and AL. The Amel α 2 subunit is found in type I and type II

KCs, but not in the ALs. The Amel α 7 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 and Grünewald 2004; Barbara et al. 2005, 2008). The nAChR is a cation-selective channel with almost equal permeabilities for Na⁺ and K⁺, and a high Ca²⁺-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 Amel α 7 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 nicotinic blockers curare, methyllycaconitine, dihydroxy- β -erythroidine and mecamlamine. 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 neonicotinoids on learning and likewise the important roles of the putatively different nAChR subtypes during learning related events were investigated (for a review, see Gauthier and Grünewald 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, 2008).

Behavioral pharmacological experiments with nAChR antagonists indicated that the nAChRs are involved in learning and memory formation. Injections of the nACh antagonists mecamlamine, α -bungarotoxin or methyllycaconitine 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).

4.2. GABA receptors

Insect GABA receptors are pentameric structures like vertebrate ionotropic GABA receptors (Jones and Sattelle 2006). The activation of honeybee ionotropic GABA receptors induces fast Cl⁻ currents (Barbara et al. 2005, 2008; Grünewald and Wersing 2008). The GABA receptors of honeybee central neurons are probably composed of RDL and LCCH3 receptor subunits with an as yet unknown stoichiometry (Grünewald and Wersing 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 Ca²⁺ (Grünewald and Wersing 2008). This modulation may be mediated via Ca²⁺-dependent phosphorylation at one of its multiple phosphorylation sites.

4.3. 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 et 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 metabotropic GluRs which may be involved during memory formation (Kucharski et al. 2007). However, electrophysiological data are missing for that receptor and only the inhibitory GluR_{Cl} of the ALs is implemented in our working model.

4.4. 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 OA receptor, AmOA1, has a widespread distribution in the honeybee brain including the AL, MB, central complex, optic lobes and suboesophageal 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, 2011). Probably, multiple OARs are expressed in the honeybee brain. Based on findings of four OAR subtypes in *Drosophila* (Evans and Maqueira 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 Ca²⁺ 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).

5. 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 octopaminergic 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 and Kreissl 1994; Goldberg et al. 1999; Wüstenberg and Grünewald 2004; Deglise et al. 2002; Thany et al. 2005). Hence, we hypothesize 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 (Figure 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

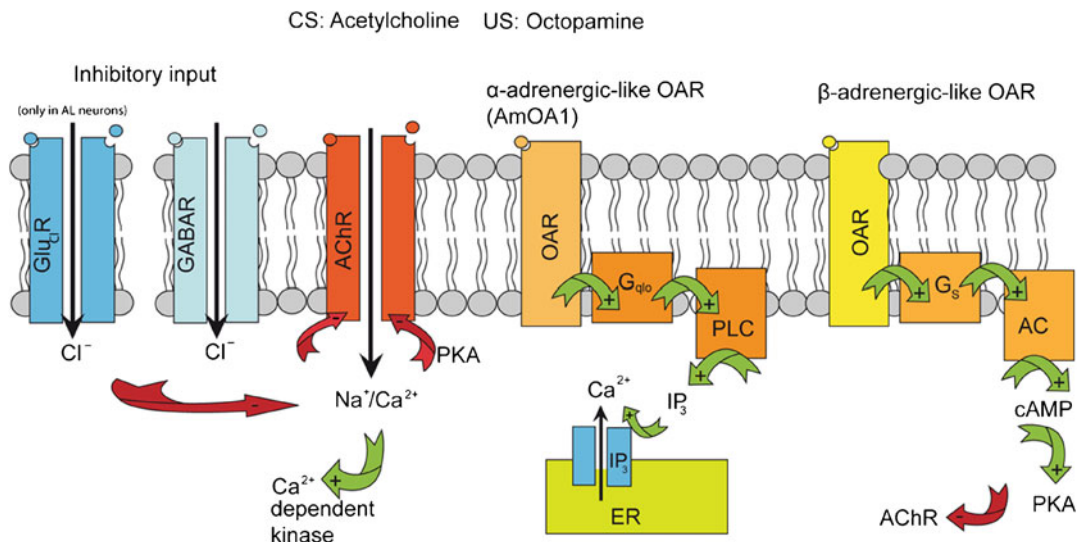


Figure 2. Schematic diagram of cellular physiology in neurons where unconditioned stimulus (US) and conditioned stimulus (CS) pathways converge. Two coincidence detectors may be assumed: (1) The increased intracellular Ca²⁺ level arises from the influx of Na⁺ and Ca²⁺ through the activated nAChR. This represents the olfactory CS pathway. The reward pathway activates either AmOA1 or β-adrenergic-like octopamine receptor (OAR). The activation of AmOA1 induces a Ca²⁺ release out of internal Ca²⁺ stores (ER endoplasmic reticulum). The AmOA1 itself is coupled to a G_q protein and a PLC which increase the concentration of inositol trisphosphate (IP₃). IP₃ in turn binds to the IP₃ receptor in the ER membrane which induces a Ca²⁺ release. This may modulate the ACh-induced synaptic currents. The elevated cytosolic Ca²⁺ level could also activate as yet unknown Ca²⁺-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 Ca²⁺ currents. (*negative and positive arrows indicate negative or positive influence of the indicated molecule/receptor.*)

the PKA *in vivo* (Hildebrandt and Müller 1995a, b) 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, 2000). (4) Olfactory learning activates a Ca²⁺-dependent PKC. Inhibition of the PKC neither affects learning nor STM or LTM, but it impairs a mid-term memory (MTM) (Grünbaum and Müller 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 in 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 Ca²⁺-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 (Figure 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 and Maqueira 2005) in the honeybee coupled to a G_q protein 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 cytoplasmic free Ca^{2+} concentration (Kamikouchi et al. 1998). The activation of the OAR (Wang and Storm 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 cytoplasmic 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.

Influx of Cl^- through GABA-/Glu-gated channels changes the intracellular electrochemical gradient which reduces 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).

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; Decourtye and Devillers 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 MB-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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Physiologie cellulaire de l'apprentissage olfactif dans le cerveau de l'abeille.

Apis mellifera / détection en coïncidence / récepteur acétylcholine / récepteur octopamine / lobe antennaire / corps pédonculé

Zellphysiologie des olfaktorischen Lernens der Honigbiene.

Apis mellifera / Koinzidenz-Detektion / Acetylcholinrezeptor / Oktopaminrezeptor / Antennallobus / Pilzkörper

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