Behavioral, neural and cellular components underlying olfactory learning in the honeybee

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Summary — A top-down approach as applied to learning and memory in honeybees provides the opportunity of relating different levels of complexity to each other, and of analyzing the rules and mechanisms from the viewpoint of the respective next higher level. Olfactory conditioning of honeybees exemplifies essential elements of associative learning and, in general, forms a bridge between the systems and the cellular levels of analysis. Intracellular recordings of identified neurons during olfactory conditioning play a key role in this effort. They allow testing of the assumptions made by modern behavioral theories of associative learning and provide access to cellular and molecular studies, owing to the identification of their transmitters and the peculiarities of the connectivity. Analysis at this intermediate level of complexity is particularly profitable in the bee, because essential neural elements of the associative network are known and can be tested during ongoing learning behavior. In this respect, the honeybee offers unique properties for the building of bridges between the molecular, cellular, neuronal, network and behavioral levels of associative learning.

honeybee / olfaction / classical conditioning / mushroom bodies / octopamine / protein kinase A / cyclic AMP / nitric oxide

Introduction

Classical conditioning of reflexes is a most convenient way to study the behavioral and neural mechanisms of associative learning. In the honeybee, proboscis extension reflex (PER) to a sucrose stimulus at the antennae is a reliable reflex in the context of feeding. The bee will extend its proboscis (tongue) reflexively when the antennae are touched with a drop of sucrose solution. It can be conditioned to olfactory or mechanical stimuli (conditioned stimuli, CS), even under conditions where the insect is restrained in a tube (Kuwabara, 1957) or prepared for physiological studies (Menzel, 1990). The PER and its conditioning to a CS are highly dependent on hunger-induced motivation.

Behavioral level

The associative nature of PER conditioning has been established by demonstrating that only forward pairings of CS/US sequences are effective in various control groups (unpaired CS and US, CS or US only presentations) and via differential conditioning of the two olfactory stimuli (Bitterman et al, 1983). The predictive value of the CS depends on the reliability with which it is causally related to the US. For example, repeated exposure to the CS in unpaired trials reduces its acquisition in subsequent conditioning. In differential conditioning, the reversal to the initially unpaired stimulus is slower after more frequent initial unreinforced pre-exposure than after a lesser number of pre-exposures. The same applies for US-only pre-exposures in an otherwise reinforced context. Partial reinforcement schedules have little effect on the acquisition function, because extinction trials do not alter the CR probability but increase the resistance to extinction and other measures of learning (Menzel, 1990).

Blocking and overshadowing experiments are used to characterize the informational content of the CS. In PER conditioning, olfactory stimuli overshadow mechanical stimuli. Blocking occurs in binary mixtures of odors (Smith and Cobey, 1994). Second-order conditioning is a procedure which tests whether a CS can acquire the potential of an US. This has also been demonstrated for olfactory PER conditioning (Bitterman et al, 1983; Menzel, 1990). The strength of the effect is highly dependent on the CS used.

Sequential memory processing

A stable life-long memory is formed even after only a few learning trials. A single learning trial leads to a biphasic retention curve with a retention minimum around 3 min after the learning trial, and an improvement of retention during the following 5 min. The early memory phase in the minute range is particularly susceptible to both extinction and reversal learning, whereas the consolidated memory is much more resistant. Amnestic treatments (cooling, narcosis, weak electroconvulsive brain stimulation) erase the memory trace if applied within less than 3 min after the single-trial learning. Several trials within a minute or longer time intervals (3 min) after a single trial render the memory trace immune to amnestic treatment. Local cooling of the peripheral olfactory neuropile (antennal lobes)
within 1 min after the learning trial causes retrograde amnesia, whereas cooling of the mushroom bodies (mb) initiates retrograde amnesia with the same time course as the whole insect suggesting that the mb's are the essential structure to establish a long-lasting amnesia resistant memory (Erber et al., 1980).

The model arising from these results localizes the associative components of the olfactory memory trace in both the mb and the antennal lobes (Menzel and Müller, 1996). The antennal lobe appears to contribute to the formation of the memory trace only during a very short period after conditioning, whereas the mb is essential for the consolidation of the trace over a longer period. Since the mb's are substrates for intense multisensory integration at the highest level of the insect nervous system whereas the antennal lobes are the primary sites of olfactory coding, both structures may be involved in different aspects of memory. The antennal lobes may store stimulus-related associative memory-components and the mb context-dependent and multisensory aspects of memory.

**Neural level**

At the circuit level, three neural structures participate in processing of olfactory information, the antennal lobe, the primary olfactory neuropile, the mushroom bodies (mb's) that receive inputs from various sensory modalities and have higher integrative functions (Erber et al., 1980, 1987; Menzel et al., 1994), and the lateral protocerebral lobe (lpl). The lpl receives olfactory information from the antennal lobe and mb-processed information (Mauelshagen, 1993; Rybak and Menzel, 1993) and presumably provides brain output that drives motor centers in the ventral nerve cord. Reward-related information is conveyed to these neuropiles by a single identified neuron, the VUMBx1-neuron, since it is activated by primary rewards and innervates the antennal lobe glomeruli, the calyces, the input region of the mb's, and the lpl (Hammer, 1993). This neuron belongs to a particular class of neurons of the ventral nerve cord in insects that have a striking bilateral-symmetric morphology, the dorsal and ventral unpaired median neurons (DUM and VUM neurons), and express octopaminergic immunoactivity (eg Bräunig, 1991; for review see Stevenson and Spörhase-Eichmann, 1995). In bees, VUM neurons of the sub-esophageal ganglion that ascend into the brain are also most likely octopaminergic (Kreissl et al., 1994). Excitation of the VUMBx1 neuron following an olfactory stimulus is sufficient to initiate behavioral associative olfactory learning. If, however, an odor is presented during excitation of VUMBx1 no learning-related behavioral changes occur (Hammer, 1993). This suggests that cellular and molecular mechanisms involved in detecting temporal contiguity between VUMBx1 and odor related neural activity must be time-sensitive.

The finding that the VUMBx1 neuron converges in three brain sites onto odor-processing neuropiles requires reevaluation of their role in associative memory induction. In Drosophila, irreversible lesioning experiments implicated the mb's (Heisenberg et al., 1985; de Belle and Heisenberg, 1994). Moreover, in Drosophila, gene products of the biochemical learning mutants dine and rut, defective in the cAMP pathway, are enriched in the mb's (Nighorn et al., 1991; Han et al., 1992; Davis, 1993). We find in bees that both the antennal lobes and the mb's contribute to associative learning. Reversible blocking experiments, as described above, can show that a certain period of undisturbed neuronal activity is necessary for memory formation in, or downstream of, the affected structure. To test which of the three VUMBx1 targets are sufficient for associative learning, its putative transmitter octopamine, was locally injected in substitution for the reward in olfactory conditioning (Hammer and Menzel, 1994). Octopamine effectively replaces the reward when injected into the antennal lobe or the mb-calyces suggesting that both neuropiles directly participate in the induction of an associative memory trace. These results support the model presented above on sequential memory processing in the bee brain, and add the important feature that olfactory memory may be established in the antennal lobes and the mb's also independently from each other (Hammer and Menzel, 1995).

**Cellular and molecular level**

Although the prominent role of the mb in insect learning and memory has been known for some time (see above), the voltage and transmitter activated currents of the mb intrinsic neurons (Kenyon cells) were not studied so far. Therefore, we examined the ionic currents of the honey bee Kenyon cells by whole cell voltage clamp experiments (Schäfer et al., 1994). The goal of the study was to test whether transmitters known to be involved in processes related to olfactory learning in honey bees (Bicker and Menzel, 1989) show modulatory activity on Kenyon cell currents. We focused on the modulation of a shaker type A-current. The A-type current turned out to be a member of the shaker potassium current family. This was proved by its kinetic properties that are unique for this current family. It has a double exponential time course for the recovery from inactivation (τ1 = 9.25 s; τ2 = 315 ms), the slow time constant depending on the extracellular potassium con-
centration. The slow time constant for the recovery from inactivation leads to cumulative inactivation under repetitive stimulation of the current. This means that the A-current integrates neuronal activity over a longer time period (several times the slow recovery from inactivation time constant) and thus may play a key role in synaptic integration and spike broadening (Ma and Koester, 1995).

Modulation of the A-current was shown to cause substantial changes in neuronal firing rates and patterns (Harris-Warrick et al., 1995). We identified several transmitters and neuromodulators that influence the kinetic parameters of the Kenyon cell A-current. These transmitters and neuromodulators (eg octopamine) are known to effect olfactory learning, memory formation and memory retrieval (Menzel et al., 1993b). In addition we gathered clear evidence that the cAMP/PKA pathway is involved in some of the observed modulations of the A-current. Mutations of both the shaker gene locus and the cAMP/PKA pathway in Drosophila generate independently a loss of performance in olfactory learning and memory (Cowan and Siegel, 1986; Davis, 1993). We conclude that modulation of the shaker-type A-current in Kenyon cells may contribute to the plasticity of these neurons in particular.

The three sites of convergence of the CS and US pathways, namely the antennal lobes, the lateral protocerebral neuropile and the mushroom bodies, express high levels of cAMP-dependent protein kinase (PKA). In the antennal lobes, the cAMP cascade is implicated in the processing of the US pathway. Application of the US causes a rapid and transient increase of PKA activity, whereas application of the CS has no effect (Hildebrandt and Müller, 1995a, b). This transient PKA elevation is mediated via the octopamine-cAMP system. Thus, the octopamine-immunoreactive VUMmx1 neuron described above, which arborizes also in the antennal lobes, is a likely cellular mediator of the US evoked increase in PKA activity (Hildebrandt and Müller, 1995b). Paired application of the CS/US stimuli produces a prolonged activation of the PKA in the antennal lobes while a CS stimulation alone does not affect PKA activity, and a single US stimulus causes only a very transient increase in activity. This finding suggests that a prolonged activation of PKA might be substrate of associative olfactory learning at the level of the antennal lobes. This might apply also for the mushroom bodies, but PKA-assays have failed so far to identify a US or CS/US-pairing related increase of PKA activity in Kenyon cell somata.

Besides classical transmitters, nitric oxide (NO) was recently identified in the brain of honeybee, and its implication in associative and non-associative learning has been demonstrated. NO synthase is concentrated in the antennal lobes and the lip region of the mb calyces, while visual neuropiles exhibit very low levels of NO synthase (Müller, 1994). Local inhibition of the NO synthase or the molecular target of NO, the guanylate cyclase in the antennal lobes specifically prevent habituation but leave unaffected neuronal processing of single chemosensory stimuli or US induced sensitization (Müller and Hildebrandt, 1995). Thus, the NO-cGMP system in the antennal lobes appears to be involved in non-associative adaptive mechanisms during chemosensory information processing.

Recent findings indicate an additional specific function of the NO system in memory formation. We know from behavioral experiments that single and multiple learning trials lead to different forms of memories (see above), a single trial to a medium term memory, repeated trials to a long-term memory lasting for days. Blocking of the NO synthase during associative learning impairs memory formation induced by multiple trial conditioning, but does not interfere with acquisition, retrieval of memory or memory formation after a single trial-conditioning (Müller, 1996). Since NO seems to act on its target in an activity dependent manner, the specific role of NO in formation of memory after multiple learning trials is probably due to a change of synaptic activity induced by the preceding conditioning trial. The molecular traces of the preceding trials have yet to be identified, but preliminary evidence indicates that NO modulates different components of the cAMP cascade. Future analyses have to show whether the cAMP cascade and/or other second messenger pathways are the target of NO in its action to transfer an early form of memory into a more lasting form.

A separation between a more labile and a stable form of memory can also be reached by massed and spaced conditioning trials. The former leads to a faster decay over days after conditioning, the latter to a permanent memory lasting many days. We found, recently, that the formation of permanent memory can be inhibited by transcription blockers (eg actinomycin D), whereas the more labile form of memory appears unaltered. This result may explain a puzzling finding reported earlier (Wittstock et al., 1993; Menzel et al., 1993a; Wittstock and Menzel, 1994) that the translation blocker cyclohexamide does not effect long-term memory. The conditioning procedures applied in these studies were rather similar to massed conditioning. It is thus likely that in bees as in Drosophila (Tully et al., 1994) protein synthesis is required only for a form of memory which results from spaced learning trials.

Conclusion

Honeybees offer the unique opportunity to study learning and memory at the mechanistic level using the same
in vivo preparation for behavioral, neurophysiological, cellular and molecular studies. Combined with the fortunate characteristics of the insect nervous system (large identifiable neurons, compartmentalized CNS, robust CNS preparations, easy handling and dissection), it is hoped that the essential components of associative learning and memory formation can be traced to particular circuits which serve this function also in unrestricted learning behavior.

References

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