Review/Model Systems Series

Searching for the Memory Trace in a Mini-Brain, the Honeybee

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To determine general or species-specific properties in neural systems, it is necessary to use comparative data in evaluating experimental findings. Presented here are data on associative learning and memory formation in honeybees, emphasizing a comparative approach. We focus on four aspects: (1) the role of an identified neuron, VUM_{mx1}, as a neural substrate of appetitive reinforcement; (2) the sequences of molecular events as they correlate with five forms of memory stages; (3) the localization of the memory traces following appetitive olfactory learning; and (4) the brief description of several forms of complex learning in bees (configuration in olfactory conditioning, categorization in visual feature learning, delayed matching-to-sample learning, and latent learning in navigation). VUM_{mx1} activity following the conditioned stimulus odor is sufficient to replace the unconditioned stimulus, and VUM_{mx1} changes its response properties during learning similarly to what is known from dopamine neurons in the basal ganglia of the mammalian brain. The transition from short- to mid- and long-term forms of memory can be related to specific activation of second messenger cascades (involving NOS, PKA, PKC, and PKM) resembling general features of neural plasticity at the cellular level. The particular time course of the various memory traces may be adapted to the behavioral context in which they are used; here, the foraging cycle of the bee. Memory traces for even such a simple form of learning as olfactory conditioning are multiple and distributed, involving first- and second-order sensory neuropils (antennal lobe and mushroom bodies), but with distinctly different properties. The wealth of complex forms of learning in the context of foraging indicates basic cognitive capacities based on rule extraction and context-dependent learning. It is believed that bees might be a useful model for studying cognitive faculties at a middle level of complexity.

Learning and Memory in a Mini-Brain

Neuroscience needs a multitude of model systems. Practical reasons favor the study of very few, probably <100 species, of the two million and more animal species for in-depth studies of brain mechanisms and the relationship between brain and behavior. Although this strategy of focusing on a rather small selection of potentially interesting and practically useful species has certainly been one of the reasons for neuroscience's success, it carries two dangers: of interpreting species-specific solutions as general mechanisms, and of overlooking the richness of mechanistic implementations for solving similar environmental demands developed during evolution. We learn from similarities and differences when we compare species, and we only recognize general mechanisms when we discover them all over again. As long as we deal with basic molecular and cellular properties of neural functions we are rather safe in assuming widespread use across species, but sensory, motor, and cognitive functions of even low complexity may be strongly adapted to the species' ecological niche, and thus reflect different solutions. Conversely, neural systems that effectively solve common demands in animal life may be conserved in phylogeny, or reinvented because of similar selective pressure. Again, such generalities can only be unraveled by comparing different species.

There is an additional motivation for comparative studies of cognitive brain functions. Human cognitive faculties are traditionally explained by referring to higher-order, declarative properties because this is the way they are accessible to our mind through introspection. In doing so we may underestimate the richness and the power of non-declarative, automatic cognitive modules and in this way may be blind for the specific additive properties of declarative functions.

I shall focus on the honeybee (*Apis mellifera*), a mid-sized insect with a brain as small as 1 μL containing 950,000 neurons (Witthöft 1967). Because of space limitations I shall select from the voluminous literature a few of the more recent studies performed in my lab. The practical advantages of using this animal as a model in neurobiological studies lie in the fact that it has a rich behavioral repertoire that is easily accessible by appetitive training. Its social life also offers a great advantage, because a nearly unlimited number of genetically close related (sisters in one colony) and equally motivated animals are available at little cost throughout the year. In the colony, bees communicate the location of a feeding place, making it easy for the experimenter who works with free-flying bees to attract new test

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animals to his experimental setup at any time. Appetitive learning is a robust and fast phenomenon in bees, making it possible to also study learning and memory in the laboratory under conditions that allow single neuron recording or optophysiological registrations of brain activity when the animals learn and remember. In addition to these favorable practical aspects, the fascination of working with honeybees comes with their impressive individual and social behavior as well as their capacity to navigate over long distances, to develop concepts of visual objects, to consider the number of landmarks passed, to form lifelong memories, to organize their decisions during a foraging trip according to optimization principles, and to communicate attractive feeding sites or nest sites to hivemates (Frisch 1967; Seeley 1995; Menzel et al. 1999; Menzel and Giurfa 2001).

A disadvantage of honeybees as a model system is that they cannot be raised outside the colony, which makes it impossible to create behavioral mutants. Transgenic animals will be of use only when inducible promoters are available, and here we still have a long way to go because knowledge of molecular genetics in bees is scarce. Molecular genetic approaches are also hindered by the lack of knowledge about the bee genome. Furthermore, and unfortunately, electrophysiological recordings from the brain are notoriously difficult as compared to bigger insects and sliced mammalian brains. However, such recordings can be extremely rewarding if they do succeed, as their value lies in the fact that it is not just an isolated brain being studied, but a brain with a body attached, actively sensing and behaving (Hammer and Menzel 1995).

The Value System in Honeybee Olfactory Learning

Reward learning is a robust phenomenon in honeybees. The preparation we use to study reward learning in the laboratory was introduced by Kuwabara (1957), who first studied color learning, and then by his student Takeda (1961), who discovered that bees restrained in tubes form an association between an olfactory stimulus and a sucrose reward. Each bee is harnessed in such a way that it can move only its antennae and mouth parts (mandibles and proboscis) freely. The antennae are the main chemosensory organs. When the antennae of a hungry bee are touched with sucrose solution, the animal reflexively extends its proboscis to reach out toward the sucrose and lick it. Odors or other stimuli to the antennae do not release such a reflex in naive animals. If an odor is presented immediately before sucrose solution (forward pairing), an association is formed which enables the odor to trigger the proboscis extension response (PER) in a successive test. This effect is clearly associative and involves classical, but not operant conditioning (Bitterman et al. 1983). Thus the odor can be viewed as the conditioned stimulus (CS) and the sucrose solution as the reinforcing, unconditioned stimulus (US). Acquisition is fast (only one associative trial leads to a conditioned response in more than half of the animals in a group), and the asymptotic level is as high as 80%–90%. Many conditioning paradigms have been tested using this preparation, and the performance exhibited by the bees resembles those found in mammal conditioning, e.g., differential conditioning, second-order conditioning, sensory preconditioning, blocking, negative and positive patterning, and inhibitory conditioning (Bitterman et al. 1983; Menzel 1990; Smith and Abramson 1992; Smith and Cobey 1994; Gerber and Ullrich 1999; Müller et al. 2000; Menzel and Giurfa 2001; N. Deisig, H. Lachnit, F. Hellstern, and M. Giurfa, in prep.).

Using this preparation, Hammer (1993, 1997) identified a single neuron that serves reinforcement during olfactory conditioning (Fig. 1). His approach was to record intracellularly from this particular neuron, the ventral unpaired neuron no. 1 of the maxillary neuromere, VUM_{mx1}, and substitute intracellular stimulation of the neuron during olfactory conditioning for the sucrose reward. Such a substitution produces approximately the same proboscis response as if the animal had been stimulated with sucrose: After a single forward-pairing of odor and VUM_{mx1} excitation, the animals' responses to odor were just as strong as those of animals that had been trained with a forward-pairing of odor and sucrose. The control for both situations was backward-conditioning, which did not lead to a conditioned response. The results thus show that VUM_{mx1} constitutes the neural correlate of the US in associative olfactory learning. VUM_{mx1} belongs to a group of neurons that are immunoreactive to octopamine, a fact that can be exploited for localizing the memory trace in the bee brain (see below).

Value systems such as the dopamine neurons of the ventral striatum and substantia nigra in mammals (Schultz et al. 1997) implement properties that propose their actions as neural correlates of reward predictors. Similar properties can be found in the VUM_{mx1} neuron (Fig. 1B,C). Intracellular recording of VUM_{mx1} during differential conditioning to two odors, a forward-paired one (CS^+: carnation), and a backward-paired one (CS^-: orange) indicate that during the course of conditioning of the CS^+ VUM_{mx1} already begins to respond at the onset of the CS^+ but not to the CS^-. Later, the response to the CS^+ resembles the original response to the US, a fact that might support second-order conditioning: If a new CS were followed by the learned CS^+, it could be transitively associated with VUM_{mx1} excitation. Furthermore, if the US follows the presentation of the learned CS^+, the response of VUM_{mx1} to the US is greatly reduced, and even inhibited. In contrast, VUM_{mx1}'s response to the US after presentation of the CS^- remains normal. This indicates that differential conditioning leads to different reward-related responses, depending on whether the reward is expected (after CS^+) or not (after CS^-). Asymptotic acquisition of CS^+ and the blocking phenomenon are believed to be behavioral indicators for US expectancy (Rescorla and Wag-

ner 1972; Rescorla and Holland 1982). Thus, similar to basal ganglia dopamine neurons, the VUM_{mx1} may be the neural substrate underlying these behavioral properties (Schultz et al. 1997).

It is not yet known whether VUM_{mx1} or the other 14 identified VUM neurons of the suboesophageal ganglion belong to a general modulatory pathway also subserving nonassociative forms of plasticity and/or associative learning of other sensory modalities (Hammer and Menzel 1994). VUM_{mx1}'s morphology makes it likely that this neuron is dedicated to olfactory learning, because it converges with the olfactory pathway at three sites: the antennal lobe (al); the lip region of the mushroom bodies (mb), an input area of the mb devoted to olfactory processing; and the lateral horn (lh), one of the premotor output regions of the brain (see Fig. 1A). Irrespective of these aspects, the predictive coding properties of VUM_{mx1} have been used successfully to model bees' foraging behavior (Montague et al. 1995).

Multiple Memories

Reward learning in honeybees initiates a sequence of memory phases that lead to long-lasting memory passing through multiple forms of transient memories (Menzel and Müller 1996; Menzel 1999; Fig. 2). An associative-learning trial induces an early form of short-term memory (eSTM) in the seconds range. This memory is highly dominated by appetitive arousal and sensitization. Thus eSTM is rather unspecific and imprecise. At the cellular level, stimulus association is reflected in the convergence of excitation of the CS pathway via nAChRs in the antennal lobe and the mb, and the US pathway of the putatively octopaminergic VUM_{mx1}, most likely acting on OA II receptors. In the antennal lobe, both cAMP/PKA and Ca^{2+}-dependent PKC are up-regulated, and the cAMP/PKA signaling cascade appears to be indicative of the associative component, because the time course of PKA activity is prolonged selectively for CS/US forward pairing (Müller 2000). However, blocking the cAMP/PKA pathway does not interfere with the associative processes and memory until a mid-term form is reached. Therefore, the cellular correlates of acquisition and consolidation after a single learning trial are still unknown ("?" in Fig. 2).

The transition to the selective associative memory trace in mid-term memory (MTM) is a rather slow process after a single learning trial lasting up to several minutes. Consolidation makes the newly established memory trace unsusceptible to retrograde amnesic treatments, and more resistant to controversial information (Menzel 1979; Gerber et al. 1998). Learning the context (visual and mechanical stimuli associated with the animal's transfer into the experimental setting) modulates the process of consolidation. If the context has already been learned, consolidation is facilitated and no longer proceeds through the dual-phase time course (Gerber and Menzel 2000). These results indicate that memory processing during consolidation depends on relevant information already stored. At the cellular level, activity in the cAMP/PKA pathway is specifically prolonged (Müller 2000).

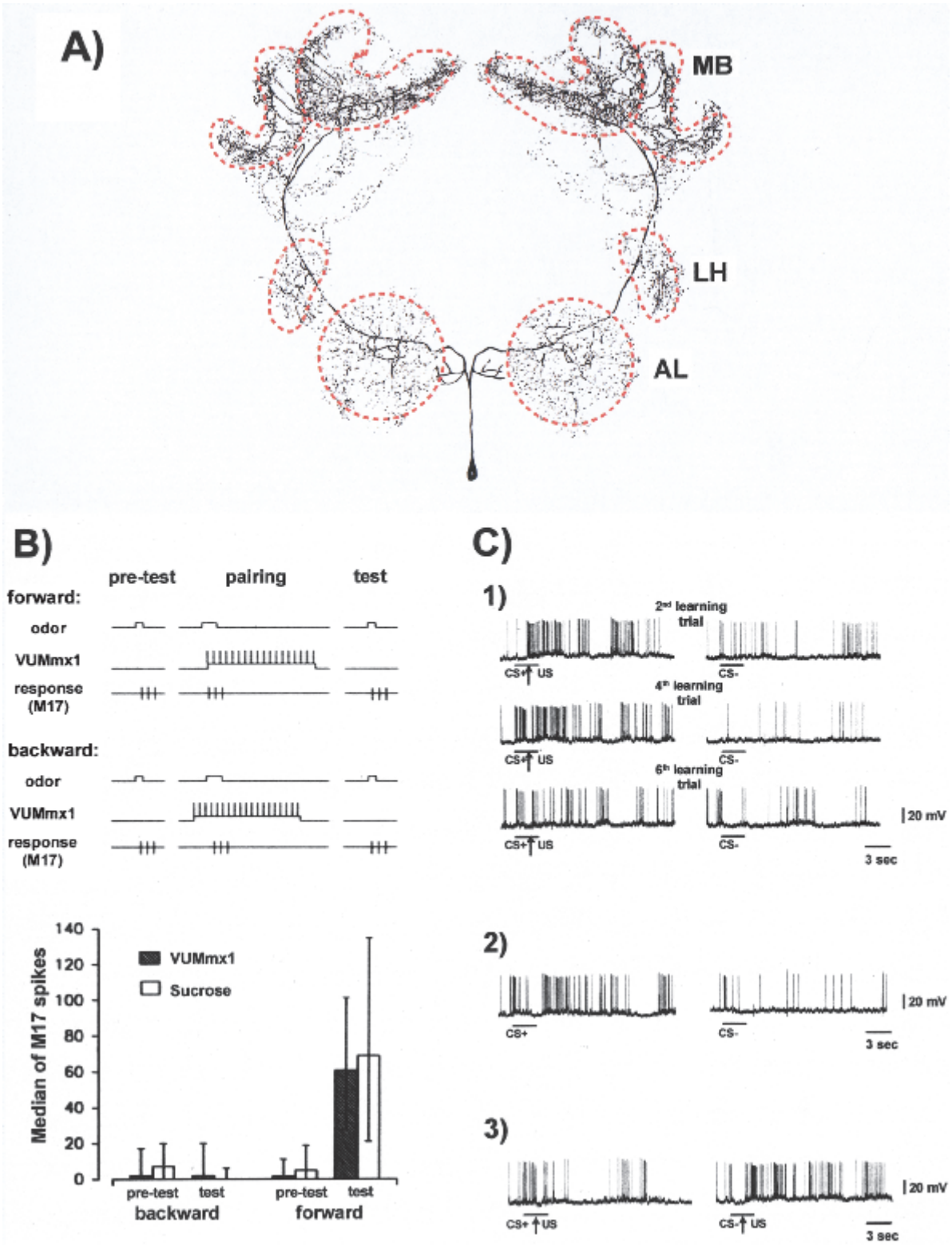
Multiple learning trials facilitate memory consolidation, leading to an unsusceptible late form of STM (lSTM) immediately on trial repetition. Thus early consolidation is both time- and event-dependent, where events must be associative experiences and not just CS or US repetitions (Menzel and Sugawa 1986). Such a dependency indicates that only the molecular substrates of associative events facilitate the transition to a stable, unsusceptible and more specific memory trace. These events are related to stronger and longer-lasting PKA activation in the antennal lobes during olfactory acquisition (Müller 2000).

Because single and multiple learning trials lead to different long-term forms of memory (LTM, see below) one can ask whether boosting PKA in the antennal lobe is a necessary and sufficient requirement for LTM formation. Two lines of evidence support this conclusion (Fig. 2): (1) Blocking NO synthase during lSTM reduces PKA activity and impedes LTM formation (Müller 1996); (2) enhancing PKA activity by uncaging cAMP in the antennal lobe after a single learning trial facilitates the formation of LTM in the same way as multiple trials do (Müller 2000; Fig. 3C).

At the beginning of MTM, behavior is controlled by consolidated, highly specific memory. After multiple learning trials, MTM is physiologically characterized by a first wave of PKC activity, whereas after a single learning trial PKC activity is not enhanced during MTM (Grünbaum and Müller 1998). The constitutive activation of PKC is a proteolytic formation of PKM that lasts for several hours. Inhibition of proteases in the whole brain by E64 reduces the formation of PKM and blocks retention during the MTM phase. However, LTM formation is not blocked by E64, indicating that protein synthesis-dependent LTM and high levels of long-lasting PKC activity (until the third day after conditioning) are formed in parallel to PKM-dependent MTM.

Mechanisms underlying memory in honeybees lasting >1 d have been an enigma. Protein synthesis inhibition does not lead to impaired retention after 24 h (Menzel et al. 1993; Wittstock et al. 1993; Wittstock and Menzel 1994), but it does so for intervals ≥3 d (Grünbaum and Müller 1998; Wüstenberg et al. 1998). Thus a memory phase usually believed to reflect LTM (24 h memory) is not protein synthesis-dependent in bees. Thus it is possible to distinguish between two forms of LTM: early LTM (eLTM, 1–2 d) characterized by protein synthesis-independent retention, and late LTM (lLTM, ≥3 d) characterized by protein synthesis-dependent retention. The time course of long-lasting PKC activity after multiple trials and its sensitivity to protein synthesis inhibition do not fully coincide with the time course of protein synthesis-dependent retention. Whereas retention is still high one and two days after conditioning and protein

synthesis inhibition, PKC activity is already reduced from the first day on, indicating that eLTM can fully compensate for the contribution of LTM lacking during this time period (Grünbaum and Müller 1998). The two forms of LTM are



formed differently, after massed and spaced multiple learning trials (R. Menzel, G. Manz, and U. Greggers, in prep.). Memory resulting from spaced trials (intertrial interval 10 min) is blocked by protein synthesis inhibitors; memory resulting from massed conditioning trials (intertrial interval 30 sec) is mostly independent of protein synthesis.

Taken together, these results indicate that memory formation in honeybees follows the general dynamics of memory processing as described in other animals. The cellular substrates appear surprisingly similar both between different species (*Aplysia*, *Drosophila*, mouse, chick, man) and between different forms of memory (declarative and nondeclarative, appetitive and aversive, emotional and nonemotional [Milner et al. 1998; Rosenzweig 1998]). This has led to the assumption that the process of memory formation is determined by its underlying cellular machinery, incorporating similar switches between memory stages (e.g., cAMP, PKA, CREB-mediated transcription of downstream genes, morphological changes) and similar time courses for the respective stages (lSTM lasting minutes, MTM hours, LTM 1 d and longer). Although this concept of generality is highly attractive and well-supported, one might keep in mind that the dynamics and respective contents of memories are adapted to species-specific needs during natural behavior (Menzel 1999). In the bee, the time courses of successive behaviors during foraging appear to match the temporal dynamics of memory stages. Choices between flowers within the same patch quickly follow each other and are performed during eSTM. Choices between flowers of different patches occur after the transition to lSTM. Successive bouts are interrupted by the return to the hive; flower choices in a subsequent bout require retrieving memory from MTM. The separation between the LTMs may be related to the flowering periods of plants in a patch. Although these ecological considerations are highly speculative, they indicate, on the one hand, that sequences of natural behavior need to be examined with respect to the intrinsic properties of the neural machinery underlying memory formation. On the other hand, they emphasize the necessity of considering the results of laboratory studies on memory formation in the context of natural behavior. Only comparative studies will help us to discern which properties reflect general mechanisms and which indicate species-specific adaptations.

Memory Traces

The three convergence sites of the olfactory and reward pathway (antennal lobe, mb, lateral horn; see Fig. 1A) are potential sites of memory formation and thus potential sites for the memory trace. Retrograde amnesia can be induced if the antennal lobes are locally chilled within a minute after single-trial conditioning, and if the mb are chilled within 5–7 min after conditioning (Menzel et al. 1974; Erber et al. 1980). No retrograde amnesia effect was observed after chilling the lateral horn. Because the VUM_{mx1} is putatively octopaminergic, one can replace the US (sucrose reward) in a conditioning experiment with local injection of octopamine into any of these three sites, and find that olfactory memories can indeed be established by separate injections into the antennal lobe or the mb calyces, but no learning was found for injections into the lateral protocerebrum (Fig. 3). It thus appears that two of the three neuropils, antennal lobe and mb, are independent loci of initial formation of olfactory memory. However, these two loci differ in an important aspect: Multiple pairing of the odor stimulus with local OA injection into the antennal lobe leads to the normal acquisition function, whereas the equivalent procedure for the mb calyx does not. Rather, memory develops after the pairings in a consolidation-like stepwise process. Several processes may account for this effect. One possibility is that

Figure 1 A single neuron represents the value system in olfactory learning in the honeybee brain. (*A*) The VUM_{mx1} neuron belongs to a group of 15 ventral unpaired median neurons of the suboesophageal ganglion, and its soma is located in the maxillary neuromere. All 15 neurons differ in their dendritic arborization structure. The dendrites of VUM_{mx1} arborize symmetrically in the brain and converge with the olfactory pathway at three sites (shown by a dashed red line): the primary olfactory neuropil, the antennal lobe (AL); the secondary olfactory integration area, the lip region of the mushroom bodies (MB); and the output region of the brain, the lateral horn (LH). VUM_{mx1} responds to sucrose solution both at the antenna and the proboscis with long-lasting spike activity, and to various visual, olfactory, and mechanosensory stimuli with low-frequency spike activity. (*B*) Behavioral learning of an olfactory stimulus can be induced by substituting the sucrose reward in olfactory conditioning of the proboscis response (PER conditioning) with an artificial depolarization of VUM_{mx1} immediately after olfactory stimulation (forward pairing). If depolarization precedes olfactory stimulation (backward pairing), no learning is observed. The same forward–backward effect is seen when sucrose is used as the reward under similar experimental conditions (Hammer 1993). In all cases the bees' response is quantified in terms of the number of spikes of M17, a muscle controlling the movement of the proboscis. The results thus show that VUM_{mx1} constitutes the neural correlate of the US in associative olfactory learning. (*C*) Intracellular recordings of VUM_{mx1} during training and tests with a reinforced (CS^+: carnation) and a nonreinforced (CS^-: orange) odor. (*1*) Intracellular recording of VUM_{mx1} during differential conditioning to two odors, a forward-paired one (CS^+), and a backward-paired one (CS^-). Such conditioning leads to an enhanced response of VUM_{mx1} to CS^+, but not to CS^-. (*2*) After differential conditioning, presentation of the CS^+ alone activates VUM_{mx1}, but presentation of the CS^- alone does not, a fact that might support second-order conditioning, a phenomenon documented in PER conditioning (Bitterman et al. 1983). In this case, if a new CS is followed by the learned CS^+, it will be transitively associated with VUM_{mx1} activation. (*3*) If the US follows the presentation of the CS^+, the response of VUM_{mx1} to the US is greatly reduced, and even inhibited. In contrast, the response of VUM_{mx1} to the US after the presentation of the CS^- remains normal. This indicates that differential conditioning leads to different reward-related responses, depending on whether the reward is expected (after CS^+) or not (after CS^-). Asymptotic acquisition of CS^+ may, therefore, result from a loss of reinforcing strength of the reward. Furthermore, this property of VUM_{mx1} is sufficient to explain the behavioral phenomenon of blocking, and may thus reflect its neural substrate (Smith and Cobey 1994; Gerber and Ullrich 1999; Hosler and Smith 2000).

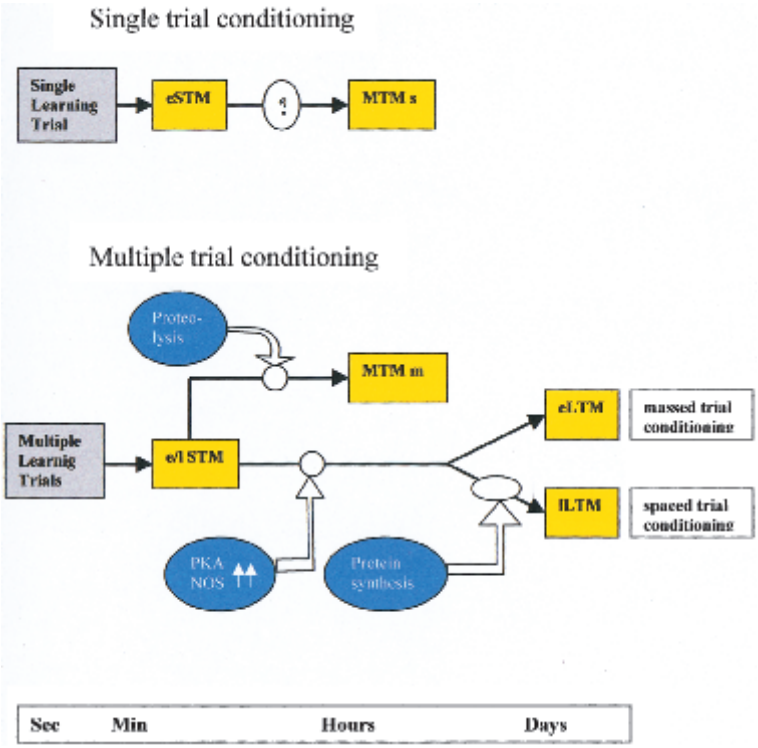


Figure 2 Model of memory phases in the honeybee. A single learning trial leads to an early form of short-term memory (eSTM) that is accompanied by a short enhancement of PKA and PKC activity. Consolidation to mid-term memory after a single trial (MTMs) is a time-dependent process lasting several minutes. The molecular and cellular events related to the transition from eSTM to MTMs are unknown. MTM decays after several hours but retention is still significant after 1 d, indicating that even a single trial can induce longer-lasting forms of memory to a low extent. Multiple learning trials lead to a succession of four memory phases that are partially arranged sequentially and partially arranged in parallel. Early and late STM (e/lSTM) are not separable, because consolidation is strongly facilitated by trial repetition, high retention rates within the acquisition process, and strong resistance to extinction and reversal trials (even immediately after conditioning). e/lSTM is accompanied by stronger and longer-lasting PKA activation and an NO synthase activation (NOS). Both cellular responses are required for the transition to LTM, but may not be necessary for MTM formation. Transition to MTM after multiple trials (MTMm) is accompanied by constitutive activation of PKM via a proteolytic pathway that is essential for MTMm formation. Blocking proteolysis, however, does not inhibit the transition to the two forms of long-term memory (LTM) indicating parallel pathways from STM to MTM and LTM. Inhibition of protein synthesis interferes only with the formation of lLTM (see text). Massed conditioning leads predominantly to eLTM, spaced conditioning to lLTM.

memory in the mb needs time to develop, whereas associative induction in the AL leads to memory much more quickly. Another possibility is that the elementary associations created in this experiment are formed only in the antennal lobe. As pairing between odor and octopamine occurred in the mb, time is required for the mb to instruct the antennal lobe about this association.

The consolidation process may be related to the particular functions of the mbs. The mbs are multisensory neuropils receiving higher-order inputs, not only from the olfactory but also from visual and mechanosensory centers. Posttraining processing (as reflected in consolidation) may have the function of establishing connections to other memories via the multisensory context under which a particular form of olfactory learning has occurred. This information is only available in the mbs, thus only the mbs can make the local and stimulus-specific memories dependent on contextual memories. Indeed, after single-trial conditioning, retention during the first three minutes, but not later, is affected by the memory of the context (Gerber and Menzel 2000). If this interpretation is correct, one would expect different memory contents in the antennal lobes and the mbs. So far no data are available on this question, but two experimental procedures have been recently established to answer it: (1) Treating honeybee larvae with hydroxyurea at an early larval stage leads to adults with partially or totally deleted mbs (Malun 1998). Exposing animals lacking both median parts of the mb to tactile learning tasks (Erber et al. 1998) reveals that elementary forms of learning are not affected, but reversal learning is (Scheiner et al. 2000). Similarly, elementary forms of olfactory learning are not affected by partial ablation of mb (D. Malun and M. Giurfa, pers. comm.). (2) The memory trace in the antennal lobe and the lip region of the mb can be visualized directly. In the antennal lobe, olfactory stimuli are coded in the spatial distribution of glomeruli excitations, and such patterns can be imaged in whole animals using Ca^{2+}-fluorescence (Joerges et al. 1997; Galizia et al. 1999, 2000). Such animals can be conditioned during the imaging process (Faber et al. 1999), and it is possible to monitor changes in the spatial distribution of odor-induced glomeruli excitation as a consequence of learning. In a differential conditioning task, the odor-induced Ca^{2+}-fluorescence intensifies for the conditioned odor (CS^+), but not for a specifically unpaired odor (CS^-). Moreover, CS^+ and CS^- activity patterns become increasingly decorrelated as a result of learning. The same effects were seen in the activity patterns recorded at the level of the lip region of the mb (T. Faber and R. Menzel, unpubl.). Further studies are needed to analyze the mechanisms of the intensified Ca^{2+} signals, and to probe for the differences between the two memory stores, particularly with respect to the role of contextual features.

Just Elementary Associations?

Having shown that honeybees constitute a useful model for the study of learning and memory of elementary associa-

tions at the behavioral, cellular, and molecular level, we may now ask whether bees might also be a suitable model for studying neural substrates for intermediate levels of cognitive complexity. The olfactory PER conditioning paradigm has also been used successfully in analyzing configural forms of learning. Rules of elementary associative learning assume that in learning a compound, animals learn separately the associations between the reinforcer and the compound elements (Rescorla and Wagner 1972). Contrary to this presumption, configural learning theories assume that, in learning a compound, animals build a new entity made from the conjunction of compound elements, and that a connection is made between this new configuration and the reinforcer (Rudy and Sutherland 1992; Pearce 1994). The various processing strategies underlying elementary and configural learning can be illustrated by paradigms in which the elementary stimuli and the configural stimuli are differentially associated with the reinforcer, e.g., in so-called patterning experiments.

In negative patterning, two single stimuli are reinforced (A^+, B^+), whereas the compound is not (AB^-). Solving this problem, i.e., responding less to the compound than to the single elements, can only be explained if configural associations are taken into account (Whitlow and Wagner 1972; Rudy and Sutherland 1992; Pearce 1994). Otherwise, summation of the elementary associative strengths in the compound should result in a stronger response to the compound than to the elements. Honeybees can solve negative patterning discrimination in olfactory conditioning of the PER (N. Deisig, H. Lachnit, F. Hellstern, and M. Giurfa, in prep.). The fact that bees can solve a negative patterning discrimination in olfactory PER conditioning shows that linear associations between single stimuli and the reinforcer are not the only ones underlying associative learning in honeybees. It will now be necessary to relate cellular mechanisms to configural forms of learning, a goal that appears to be attainable, because the PER conditioning paradigm can be used.

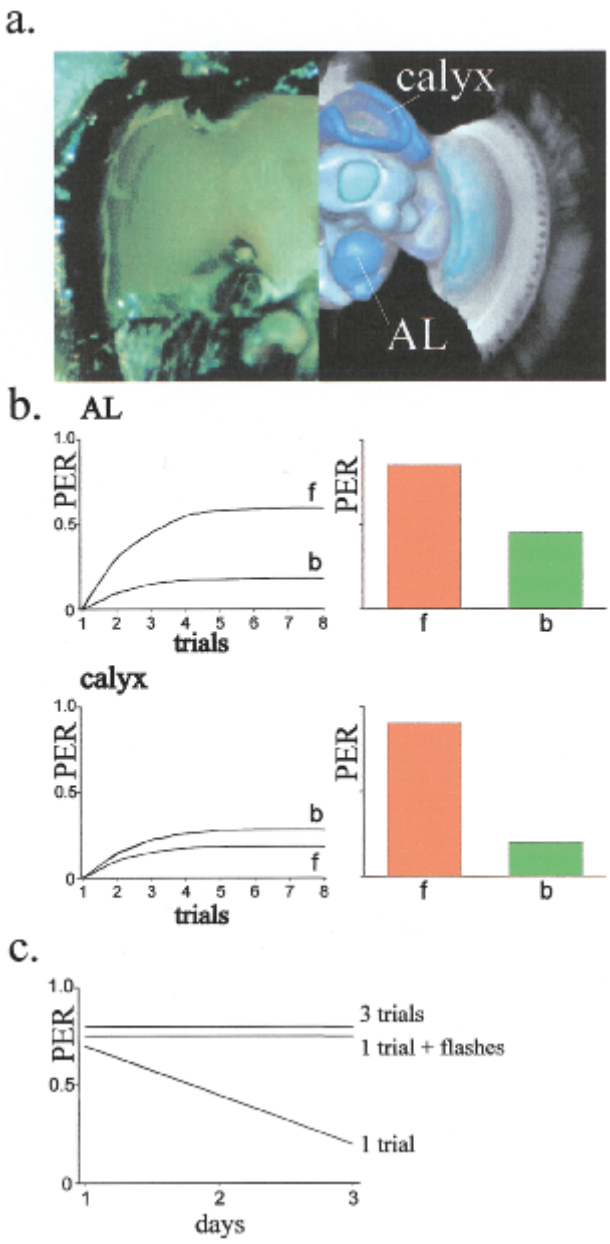


Figure 3 Probing localization of the memory trace in the bee brain. (*a*) The bee brain as it appears to an experimenter aiming electrodes toward particular neurons or a micropipette to an area in the brain for microinjection (*left*). The right side gives a 3D reconstruction, from confocal images, highlighting in blue the input region of the mushroom body, the calyx, and the primary sensory neuropil of the olfactory pathway, the antennal lobe (AL). (*b*) Local injection of octopamine as a substitute for the US in olfactory PER conditioning. Bees were stimulated with the conditioned odor on one antenna and octopamine (10–6 m) was injected in the ipsilateral antennal lobe or the ipsilateral calyx immediately afterwards (f: forward pairing of the experimental groups). In the control groups, octopamine was injected first and odor stimulation followed (b: backward pairing). Acquisition rises continuously during multi-trial antennal lobe forward-pairing, but not during calyx forward-pairing. Retention tested 20 min later is significantly higher after forward-pairing for both locations, antennal lobe and calyx (Hammer and Menzel 1998). (*c*) Transfer from short- to long-term memory by activating the cAMP/PKA pathway in the antennal lobe. A single olfactory PER conditioning trial leads to decreasing retention over several days (one trial). Three learning trials produce a stable long-term memory (three trials). If cAMP is released by flashing UV light on to the antennal lobes shortly after a single learning trial and thus releasing cAMP from a caged compound, long-term memory is also formed after a single trial (one trial + flashes; Müller 2000).

Complex Learning in a Natural Setting

More complex learning tasks can be imposed on free-flying bees. A possible question is, for example, whether bees are able to categorize stimuli, extract rules from sequential learning, and plan future behavior. Visual categorization

was studied for two kinds of patterns, vertical stripe orientation and bilateral symmetry. Bees easily learn orientation as an independent parameter (Hateren et al. 1990): If they are trained with a series of different patterns to discriminate vertical from horizontal stripes, they can transfer this information to different new patterns sharing the features vertical versus horizontal. Similarly, bees learn to extract bilateral symmetry or asymmetry from a series of different, changing patterns and transfer this information to novel symmetrical and asymmetrical stimuli (Giurfa et al. 1996). Moreover, in the case of symmetry learning, bees seem to integrate symmetry and asymmetry into a unique concept. After having learned one feature, e.g., symmetry, they do not need to relearn the alternative feature (e.g., asymmetry) anew if it is reinforced in a reversal-learning experiment, rather, they quickly choose it after the reversal. In other words, when bees learn about symmetry they also learn about asymmetry (Giurfa et al. 1998).

Rule extraction can be demonstrated with a delayed matching-to-sample task. When bees are trained in a delayed matching-to-sample task they are presented with a changing nonrewarded sample (i.e., one of two different color disks or one of two different black-and-white gratings, vertical or horizontal) at the entrance to a Y-maze (Giurfa et al. 2001). The bees are rewarded only if they choose the stimulus identical to the sample once within the maze. Bees trained with the colors and presented in transfer tests with the gratings that they have not experienced before are able to solve the problem and choose the grating identical to the sample at the maze entrance. Similarly, bees trained with the gratings and tested with colors in transfer tests also solve the problem and choose the novel color corresponding to the sample at the maze entrance. Thus bees make a judgment regarding "sameness" among objects in their environment. Comparable results are obtained in a delayed nonmatching to sample task, thus showing that bees can also learn a "difference" relation between objects in their environment.

Navigating over distances of several kilometers is a formidable task for such a small insect, and bees are indeed perfect at finding their way around. The strategies underlying navigation are rather well-known, but the level of integration and the kind of learning involved is under debate. Flight distance is estimated by the visual flow field (Srinivasan et al. 1997). Celestial cues (sun, polarized light pattern) and landmarks are used to determine the rotary component of flight vectors (Frisch 1967). Picture memories are established both for important locations (hive, feeding sites) and along flight routes, and are used as a backup system on overcast days (for review, see Collett and Zeil 1998). The traditional way of studying these components of orientation by training bees along a route and then releasing them at different sites has been an adequate method for studying the independent action of these components, but has hindered research on navigational strategies. Under traditional conditions, bees always depart from the release site after route training in the direction that they would have taken if they had not been translocated, because the compass direction of the route memory dominates initial behavior at the release site and suppresses more flexible spatial memories. Using different training paradigms that avoid route training, it was shown recently that bees can navigate according to a spatial memory that allows them to return to the hive from any location around the hive within the distance of their orientation flights (a few hundred meters; Menzel et al. 2000). In this case bees were trained to forage on a feeder that rotated around the hive at a small radius. As a consequence they did not establish a route memory and were guided by a memory acquired by latent learning during their exploratory flights. This "general landscape memory" is suppressed by the route memory, and bees can refer to it only when they have not established a route memory or have used it, but have not yet arrived at the goal. These new results on navigation indicate a highly flexible form of spatial memory, allowing the bee to travel to an intended goal along novel shortcuts. This goal does not need to be the central spot at which all excursions originate (the hive) but can also be a transitory location such as a feeding place (R. Menzel, J. Riley, and U. Greggers, unpubl.). One might, therefore, ask what bees are indicating when they communicate a rich food source or a potential nest site by their waggle dance (Frisch 1967) — the direction and distance of a flight toward it, or the location in a neural representation of space. In the latter case they might rely on some form of planning when they decide to follow the instructions gathered from the dancing hive mate.

Conclusion

The honeybee provides a model system for the study of neural substrates of low and intermediate levels of cognitive faculties. Neural analysis is supported by robust forms of associative learning that occur even under conditions where intracellular recordings or optophysiological measurements of brain activity are performed. The functional organization of the brain, with a considerable number of uniquely identifiable neurons, is also advantageous for relating cognitive functions to neural events in circumscribed circuits. However, the battery of behavioral functions that can be tested under such constrained conditions needs to be extended, and methods for monitoring neural correlates at the single-neuron and circuit level must be improved. Molecular genetic techniques will eventually allow us to express or block expression of particular genes in particular regions of the brain under conditional control, thus overcoming the problem of a social animal, but such methods certainly lie far in the future, as very little is known cur-

rently about the bee genome, and nothing at all about promoters and their selective action in neurons or brain regions. The fascination in working with bees comes from their impressive behavioral repertoire, and this fascination will certainly continue to motivate generations of neuroscientists to come.

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