LEARNING AND MEMORY IN HONEYBEES: From Behavior to Neural Substrates

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ABSTRACT
Learning and memory in honeybees is analyzed on five levels, using a top-down approach, (a) Observatory learning is applied during navigation and dance communication. (b) Local cues at the feeding site are learned associatively. (c) Classical conditioning of the proboscis extension response to olfactory stimuli provides insight into behavioral, neural, and neuropharmacological mechanisms of associative learning. (d) At the neural level, the pathways coding the conditioned and the unconditioned stimulus are identified. The reinforcing function of the unconditioned stimulus is traced to a particular neuron. (e) At the cellular level, the cAMP pathway is found to be critically involved. Nitric oxide is an essential mediator for the transfer from short- to long-term memory.

INTRODUCTION
This article reviews the literature on learning and memory in honeybees, applying a top-down approach from behavioral to neural and cellular studies. We emphasize mechanistic analyses that try to relate behavioral phenomena to the neural substrate and its cellular components. Observations on freely behaving animals are also included, with the aim of relating the behavioral phenomena studied under tightly controlled conditions with those at work in the natural context. This neuroethological approach is particularly useful for studying an insect whose rich behavioral repertoire is strongly influenced by individual experience.

A Historical Note
Throughout this century the honeybee has been used as a model organism for the discovery of unknown sensory capacities in animals and to unravel the
mysteries of social interactions in a community of 50,000 to 100,000 animals. The experimental tool used in all of these studies was the training technique of single and individually marked animals, as introduced by Karl von Frisch at the turn of the century. He studied the perception of colors, patterns, and odors and developed a unique and effective method for handling bees in discrimination tasks (reviewed in von Frisch 1967). Rewarding a marked foraging bee with sucrose solution at an artificial feeding site allowed von Frisch to relate the experience of this particular bee with its choice behavior after it had systematically manipulated the signals attached to the feeding site. Using this technique, von Frisch and his colleagues discovered a whole range of sensory capacities in bees, e.g. sensitivity to ultraviolet light (Kühn 1927), trichromatic color vision (Daumer 1956), polarized light vision (von Frisch 1949) dance communication, sun compass orientation, time sense (Lindauer 1954, von Frisch 1967), sensitivity to the earth's magnetic field (Martin & Lindauer 1973), and many more (most of the work is described in von Frisch 1967). The process of learning, however, was not at the center of von Frisch's interest, and it became a research topic only much later (Lindauer 1970), although the question of which of the impressive cognitive capacities of bees are learned and which are innate was a matter of debate for decades (Buttel-Reepen 1900; Gould 1984; Lindauer 1959, 1963, 1967; von Frisch 1937).

The Biological Context

da Frisch's training technique utilized the fast and effective learning abilities of a social insect, which needs to return reliably to its nest site for brood care, protection, and shelter. The colony as a whole is an organism with a potentially infinite life span, and its members are exposed to highly variable ecological conditions during the course of the year and in different habitats. Therefore, the individuals, although living only for a short period of time, cannot be prepared genetically for the ecological conditions of any particular habitat. Because bees search for food (nectar and pollen) at unpredictable sites, they have to learn the celestial and terrestrial cues guiding them on their foraging trips over long distances (in the range of several kilometers). The social bees benefit from a highly variable flower market by adjusting their search behavior continuously, and they cannot be tuned to any close flower-pollinator relationship (Heinrich 1983, Kevan & Baker 1983, Seeley 1985).

Bees perform these tasks with a rather small brain of approximately 1 mm and fewer than 960,000 neurons (Witthoft 1967). As a result, some researchers suspect that learning is guided more closely by genetic predisposition (Gould 1984, Lindauer 1970, Menzel 1990). The search for the neural basis of the adaptive components of behavior is facilitated by the organization of the insect nervous system, which is well compartmentalized, with clear separations be-
tween multisensory higher-order neuropiles in the brain and neuropiles serving sensory-motor routines in the ventral cord. Many neurons in the insect CNS are individually recognizable and large enough to be recorded intracellularly during ongoing behavior, including learning.

LEARNING IN THE NATURAL CONTEXT

*Biology of Foraging*

Angiosperm plants provide food (nectar, pollen) at their reproductive organs, the flowers, to use insects as vectors for the transport of their genetic material (Darwin 1876, Sprengel 1793). Because outbreeding appears to be favored by evolution (Waser & Price 1983), flower fidelity on the part of the individual insect is one of the evolutionary traits developed in flower-pollinator interaction (Feinsinger 1983, Waser 1983). Rovers increase the specific pollen transfer by providing signals for localization and recognition and by offering only minute amounts of reward per flower, thus forcing the insect to visit many flowers on each foraging bout. Pollinators, for their part, gain from flower fidelity if flowers of the same species are abundant and if foraging becomes more effective and less risky by staying with one species (Heinrich 1983, Laverty 1994). Because flowers are often complicated structures that require special handling skills, flower fidelity may well be favored because of improved handling skills acquired by sticking to one kind of flower (Heinrich 1984, Laverty 1994). The costs and benefits of learning become apparent when species of bumble bees, which belong to pollinator specialists (innate preference for one plant species) and generalists (potential pollinator of many species), are compared (Laverty & Plowright 1988). The specialist always handles its flower better than the generalist, but the latter can learn new manipulatory movements and improve its performance with practice. Hymenopteran pollinators apply innate search images for selecting potential food sources (Giurfa et al. 1995, Menzel 1985) or locations of food on flowers (Daumer 1958, Lunau 1991). However, learning flower cues and flower location in space is the most important factor in foraging. Honeybees acquire a knowledge base for effective foraging by two means: exploratory experience and the information transfer during the dance performance of recruiting bees (von Frisch 1967).

*Choice Performance in Foraging*

After a patch of flowers has been found that provides enough food, the individual bee sticks to a particular kind of flower within a patch and may travel kilometers visiting up to hundreds of flowers during one foraging bout. Because the bee participates in the colony's information flow about alternative food sites (Seeley 1985, 1994), it is constantly informed about the effectiveness
of its foraging performance. Out in the field, it focuses its efforts on the most productive flowers, indicating that it evaluates and learns the reward conditions of multiple feeding sites. Bees optimize their foraging efforts by choosing flowers or artificial feeders with high rewards, as opposed to those with lower rewards (Heinrich 1984). Two strategies are applied: maximizing (choice of only one feeder) or matching (choice frequency increases with the profitability of the feeder). In a patch with up to eight artificial feeders, each of which produces a different but constant flow rate of sucrose solution, bees maximize if one feeder provides a flow rate >1 μl/min; they match if the total flow rate in the patch lies between 0.4 and 1 μl/min; and they terminate foraging for total flow rates <0.4 μl/min (Greggers & Menzel 1993). In contrast to earlier model explanations of choice behavior based on random walk assumptions (Pyke 1984, Schmid-Hempel 1986), bees develop feeder-specific memories that relate feeder signals with measures of their profitability. Accordingly, incentive contrast is a robust and long-lasting phenomenon (Couvillon & Bitterman 1984). Interestingly, colors appear to signal unconditioned stimulus (US) strength less reliably than odors (Buchanan & Bitterman 1988, Couvillon & Bitterman 1988). The most important measure of profitability is the time spent licking per visit; energy content (sucrose solution concentration) is of secondary importance (Greggers et al 1993).

An analysis of the sequences of visits within a patch of two, four, or eight feeders and of feeding activities at each visit reveals learning rules and the existence of several incentive memories. Learning performance can be formalized according to the difference rule (Rescorla & Wagner 1972, Sutton & Barto 1981) and by assuming two forms of memory, a general patch memory and feeder-specific memories. The feeder-specific memories are highly dynamic and change their content over time and as a result of new experience (Greggers & Menzel 1993). Corresponding temporal dynamics of choice behavior were found for bumble bees foraging in a natural environment (Chittka et al 1995). The probability of the bumble bee switching to another kind of flower depends on the licking times during the last visits and the time interval between choices.

**NAVIGATION** Central place foragers like the bee navigate in an egocentric reference system based on celestial compass information (e.g. sun, polarized light patterns) and route-specific landmark memories (Collett 1993, Wehner 1992). Celestial compass orientation requires the use of a time-compensated measure of the sun’s azimuth. Bees learn the full solar azimuth/time function from temporary restricted segments (e.g. when they are exposed only to the late afternoon sun), indicating that they refer to an innate template that is adjusted by the learning process (Dyer & Dickinson 1994). Wehner (1984) first suggested this form of learning, which is used by the desert ant *Cataglyphis fortis*. The celestial compass provides the reference system for the
rotatory component around the high body axis during flight, and this measure is used together with estimates of flight distances to continuously integrate the flight path (Wehner & Wehner 1990). Path integration (or dead reckoning) is a form of automatic observatory learning that enables the animal to return to the hive at any time along a straight line. Landmarks are learned within the framework of the celestial compass (von Frisch 1967) and can also be used to derive the compass direction under a fully overcast sky (Dyer & Gould 1981, von Frisch & Lindauer 1954). Landmark memories are stored as images arranged in sequences as they occur during outbound and homebound flights. Image memories are formed when the animal looks at the goal from a guide post at a selected distance and direction (Collett & Baron 1994, Collett & Cartwright 1983, Vollbehr 1975). Each image memory may be a retinotopically stable flash memory as demonstrated recently for Drosophila melanogaster (Dill et al. 1993), thus simplifying the procedure of establishing and retrieving a picture memory of a location. Observations of the flight path and model calculations support this view (Cartwright & Collett 1983, 1987). Sequences of landmark memories are established for multiple feeding places visited successively (Collett 1993) and en route when bees pass landmarks on a continuous flight towards a food source (Chittka & Geiger 1995). In the first case, bees expect the food source at a specific location relative to the surrounding landmarks; in the second, bees expect a particular sequence of, and distance between, several landmarks en route.

Landmark and cue memories are linked to time (von Frisch 1967). Bees learn to fly toward a particular feeding place at a particular time of day and to expect reward at a particular set of cues (Bogdany 1978, Gould 1987, Koltermann 1971). The vector memories attached to each feeding place are retrieved specifically by the time of day and the landmarks surrounding the feeding place. For example, marathon dancers—bees that dance for a long time or can be stimulated to dance at any time, even at night—indicate the direction and distance of the place visited closest to the actual time (Lindauer 1954). Furthermore, bees trained to two different feeding places in the morning and afternoon fly in the correct sun compass direction according to the time of the day when released at an unfamiliar place. However, if they are released halfway between the morning and afternoon places, both vector memories are retrieved and a large proportion of bees fly straight toward the hive, indicating that the two vector memories are integrated (Menzel et al. 1995a,b). Thus the navigational behavior of bees is guided by contextual and content addressable memories. Therefore, bees appear not to represent spatial memory in a geocentric map-like organization, as suggested by Gallistel (1990) and Gould (1986b). No convincing evidence yet establishes that bees are able to determine their position at a new place relative to a desired place other than by dead reckoning, whereby they steer a novel flight route according to the geometric
arrangement of landmarks (Wehner 1992, Wehner & Menzel 1990). Instead they reach partial solutions to cope with problems arising from unexpected displacement by referring to different memories in a hierarchical order.

LEARNING DURING DANCE COMMUNICATION Information transfer during dance communication has been proven beyond a doubt by using a robot bee to indicate distance and direction of a test location (Michelsen et al. 1992). The learning process of the attending bees is not yet well understood. Because dancers do not always feed the attending bees—in particular when they are indicating the locations of pollen, water, resin, and new nest sites (Seeley 1985, von Frisch 1967)—learning cannot be a type of appetitive reward learning. The acoustic pulse emitted by the dancing bee at the moment of correct body position (Michelsen et al. 1986) probably provides the necessary signal for the significant phase of the dance. In food foraging, olfactory and gustatory stimuli are exchanged between the dancing and the attending bees to reduce ambiguity regarding the source the dancer is recruiting. These stimuli are indeed learned, because a recruited bee expects, for example, a food source with the odor it had smelled during dance communication (von Frisch 1967). The probability that a recruited bee will be successful grows with the number of attended dances (Mautz 1971). The code for distance and direction need not be learned, but both the dancing and the attending bees must have learned the solar azimuth/time function and be familiar with the location of the hive. The level of complexity of cognitive functions involved in dance communication is controversial (Gould & Gould 1982). There is no convincing evidence to show that attending bees compare different dances before making a decision (Seeley & Towne 1992) or reject flying toward an impossible place (e.g. a feeding station in a lake), as claimed by Gould & Gould (1982).

APPETITIVE CUE LEARNING AND MEMORY IN FREELY BEHAVING BEES

Salient Stimuli in Operant Conditioning

Appetitive learning in freely flying bees follows some rules of operant conditioning (Grossmann 1973). Bees on fixed-ratio reinforcement perform twice as many choices as those trained on continuous reinforcement, and bees on fixed-interval schedules respond less than those on fixed-ratio schedules. The stimuli associated with reward differ with respect to their saliency. A few signals that are perceived in other contexts (e.g. polarized and flashing lights, rotating sectors) are not learned, but most signals, even those that do not resemble flower cues, are learned well, although at different rates (Menzel 1990). Color signals are a case in point. Intensity differences are not learned,
although they are perceived in spatial vision and optomotor flight control (Lehrer 1994) and phototaxis (Menzel & Greggers 1985). Spectral lights around 400-420 nm (bee UV-blue) are learned fastest; those around 490 nm (bee blue-green) slowest (Menzel 1967). Hue salience reflects an innate predisposition and can be understood as an innate expectancy of reward probability (Giurfa et al. 1995). Odor salience is also ranked according to biological significance (Koltermann 1973), but even repellent stimuli or the bee's own sting pheromone are readily learned (Menzel 1990).

Contiguity

To learn local floral cues (e.g. odor, color) as reward predicting stimuli (conditioned stimuli, CSs), bees need to perceive them at arrival just before they experience the reward (unconditioned stimulus, US) (Gould 1987; Grossmann 1970, 1971; Menzel 1968; Opfinger 1931). The optimal time interval between CS and US is a few seconds in single-trial conditioning (Menzel 1968). However, this interval depends on the training conditions. For example, the CS-US interval can be extended by introducing a secondary reinforcer (e.g. odor) (Grossmann 1971). Bees also learn at departure when they perform characteristic circling flights (Couvillon et al. 1991, Gould 1986a, Lehrer 1994, Lehrer & Collett 1994), which resemble those known from orientation flights of young bees at the hive entrance (Buttel-Reepen 1900, Vollbehr 1975) and from sand wasps at their nest sites (Baerends 1941, Tinbergen 1932). The extent of the orientation flights depends on the degree of novelty of the CSs involved and the US. Observatory learning taking place during these hovering and circling flights focuses on surrounding landmarks and increases with flight duration but appears to be independent of the US. Therefore, learning during departure is probably not a form of backward conditioning.

The Effect of Other CSs

Stimuli predicting an important outcome do not appear in isolation; they appear usually in compounds of several stimuli. In the case of appetitive learning in bees, color and odor are most important and thus were studied intensively by applying paradigms developed in experimental psychology (e.g. blocking, overshadowing, within-compound association, CS pre-exposure) (Bitterman 1988). Experiments with freely behaving bees are, however, complicated by the fact that color is a far distance signal, and odor a close-up signal. Because the bee's settling on a target is usually taken as the behavioral criterion for learning, there is strong bias in favor of the odor effects. The influence of color is hardly quantifiable in color-odor compounds, owing to the uncertainty with which color is perceived, attended to, and chosen. Using color and odor as CSs, Couvillon et al. (1983) found no blocking effects, and overshadowing (odor over color) appeared to be an unstable phenomenon. A particular color appears to be associated with the odor if the bee is rewarded with the compound
of the two stimuli (indicating within-compound association) (Couvillon & Bitterman 1982, 1988), but it is as yet unknown whether the mechanism at work is second-order conditioning (odor being the primary CS because it is at closer contiguity to the US) or whether color is a context or occasion setting stimulus for the association of odor.

**The Effects of Unpaired USs and CSs**

Additional USs not predicted by the CS have a profound effect on conditioning in vertebrates and lead to a reduction of CS acquisition due to a loss of predictive power by the CS (Rescorla 1967). Appetitive USs cannot be presented independently of CSs and contextual stimuli in freely behaving bees. In an attempt to study the effect of unsignaled USs, Abramson and colleagues (Abramson 1986, Abramson & Bitterman 1986) used an aversive conditioning procedure developed by Nunez & Denti (1970), in which the bees are taught to stop sucking sucrose solution to avoid an averse electric stimulus (see also Kirchner et al. 1991, Towne & Kirchner 1989). They found stronger aversive learning for unsignaled USs than for USs signaled by vibration or air stream. Unfortunately, these experiments were not developed to the point where it was possible to scrutinize the role of the predictive relation between contextual stimuli, the CSs, and the aversive US. Latent inhibition, the effect of unreinforced CS preexposure, was tested with the same paradigm. Subsequent conditioning was found to be retarded, indicating the possibility that CS experience, uncorrelated with the US, leads to inhibitory learning.

**Aversive Conditioning**

In this context one might ask whether aversive learning is of any biological relevance. Bees avoid, or learn to approach from the side, flowers that knock them off with a click of the petals designed for larger pollinators (e.g. Alfalfa flowers). Gould (1988) successfully trained bees to avoid strongly vibrating artificial petals. Spiders spin webs with patterns that appear to attract bees, because they might mistake them for flowers. Bees that manage to escape from them avoid them afterward (Craig 1994a,b; Craig & Ebert 1995). Spiders, for their part, try to compensate for avoidance learning by changing the web pattern (Craig 1994b).

**MEMORY DYNAMICS AND MEMORY CONTENTS**

**Time Course of Retention and Memory Consolidation**

Long-term memory (LTM) in bees can last for several months, surviving the winter rest of the colony (Lindauer 1963) and, in summer, lasting the life span of a forager (about 2 weeks)—even after only three learning trials (Menzel
A single learning trial initiates time-dependent processes, leading to high retention immediately after the trial, low retention 2-4 min later, consolidation to a high level within the next 10-15 min, and vanishing retention over several days (Erber 1975a,b; Menzel 1968). The early phase depends on the strength of the US and is highly sensitive to extinction and reversal trials (Menzel 1968, 1969, 1979). Retrograde amnesia induced by weak electroconvulsive shocks, cooling, or narcosis is most effective during this early phase (Erber 1976, Menzel 1968). The dynamics of memory are interpreted to reflect memory formation rather than retrieval processes. The model assumes that a transitional short-term memory (STM), which is susceptible to experiential and experimental interference, is followed by a slowly consolidating middle-term memory (MTM), which is more stable in the face of new experience and unaffected by amnestic treatments (Menzel 1983, 1984). Multiple trials lead to LTM, which can be established within less than 2 min, if the trials follow each other quickly. Thus, the transfer from STM to MTM and LTM is both time and event dependent. Memory dynamics in bees resemble many of those features known from studies in mammals and humans (Squire 1987). Correspondence between the early dual time course and the consolidation phase is particularly striking. As in vertebrate studies, it is difficult to distinguish between retrieval and storage phenomena in the expression of memory. In contrast to the interference theory of consolidation (Keppel 1984), modern biological concepts refer to the notion that what is stored can change; thus consolidation is likely to reflect memory formation processes (McGaugh & Herz 1972). The same arguments have been applied to the bee on the basis of physiological data and more tightly controlled behavioral experiments (Hammer & Menzel 1995) (see also below).

OLFACTORY CONDITIONING: BEHAVIORAL ANALYSIS

The Olfactory Conditioning Paradigm

PROBOSCIS EXTENSION RESPONSE Many insects extend their tongues (proboscis) reflexively when the sucrose receptors at the antennae, mouth parts, or tarsae are stimulated. Kuwabara (1957) and Takeda (1961) found that the proboscis extension response (PER) of bees can be conditioned to visual and olfactory stimuli if the bees are allowed to suck sucrose solution following the presentation of these CSs. Odors are associated with sucrose much faster (Menzel et al. 1974, Vareschi 1971) than visual stimuli (Masuhr & Menzel 1972). PER strength is expressed by the probability of PER in a group of animals equally treated or in graded response values, such as the number of muscle spike of the muscle M17, the latency or duration of PER or muscle...
spikes, the number of licking movements of the glossa, and other measures (Rehder 1987, Smith & Menzel 1989a,b).

CLASSICAL CONDITIONING OF PER Olfactory PER conditioning is a typical case of classical conditioning, with many features known from vertebrate literature. These aspects were reviewed more recently by Bitterman (1988), Menzel (1990), Menzel et al. (1991, 1993b), and Hammer & Menzel (1995). A single pairing of the odor as the CS with sucrose as the US changes the PER probability from a spontaneous level of usually ≤10 to a level of ≥60% and multiple trials to an asymptotic level of ≥80%. Timing of CS and US presentation during the conditioning trial is an essential requirement. Forward pairing (CS precedes US by 1-3 s) is most effective. Backward pairing or unpaired CS and US presentations do not change PER probability (Bitterman et al. 1983), and an inhibitory component is uncovered by the retardation of acquisition during subsequent forward pairing (Menzel 1990). The inhibitory effect is strongest for intermediate US-CS intervals and low for short and long US-CS intervals (Hellstern & Hammer 1994). Excitatory and inhibitory conditioning are combined in differential conditioning leading to a high (≥80%) PER probability to the CS+ and zero to CS−. Because bees generalize between the CSs, the response to CS− is initially high and decreases when learning progresses. Conditioned PER also develops in the absence of the US, e.g. in the case of second-order conditioning (Bitterman et al. 1983, Menzel 1990). Furthermore, conditioning may be prevented or reduced even with CS-US forward pairing, e.g. in blocking and overshadowing paradigms (Rescorla & Holland 1982). Blocking between two odors was convincingly demonstrated by Smith & Cobey (1994), and overshadowing occurs in olfactory-tactile compound conditioning (Bitterman et al. 1983).

NONASSOCIATIVE COMPONENTS OF PER The appetitive stimulus, sucrose, arouses the animal and transiently sensitizes feeding-related responses, e.g. probability and strength of PER (Menzel et al. 1991). Sensitization depends on the number and duration of sucrose stimulations and the site of stimulation (antennae, proboscis, or both as a compound) (Hammer et al. 1994; Menzel 1990; Menzel et al. 1989, 1991). For example, three compound stimulations are less effective than one. A long proboscis stimulation is more effective than a short one, but a long antennal stimulation is less effective than a short stimulation. These and other results were compiled in a model that assumes that during sucrose stimulation, bees acquire specific properties of the sensitizing stimulus by employing excitatory and inhibitory forms of learning, depending on the site of input, its frequency, and its duration (Hammer et al. 1994).

Habituation to multiple sucrose stimulation at the antennae develops quickly
for low concentrations of sucrose and slowly for high concentrations (Braun & Bicker 1992). Habituation is restricted to the input side, and stimulation of the contralateral antenna with high concentrations dishabituates the animal. A single session of habituation trials leads to a short-lived effect (≤10 min), multiple sessions to longer-lasting effects (about 24 h) (Bicker & Hähnlein 1994).

Variations and Applications of PER Conditioning

AVERSIVE PER CONDITIONING Bees quickly learn to discriminate between two odors; one of which is paired with sucrose, the other with sucrose plus electric shock (Smith et al. 1991). PER probability to the shock-paired odor is not only much lower, but PER to sucrose is withheld or delayed in the context of this odor. The response strategies during PER conditioning appear to differ considerably between individuals, suggesting that aversive conditioning recruits different preparatory responses in different groups of animals, possibly with respect to their genetic background, age, and ethotype (actual duties in the colony).

OLFACTORY DISCRIMINATION AND KIN RECOGNITION BY CHEMICAL CUES Olfactory PER conditioning has been successfully used to study olfactory discrimination (Smith & Getz 1994, Vareschi 1971) and kin recognition (Getz & Page 1991, Getz et al. 1986). Bees can also be trained to nonvolatile chemicals (e.g. cuticular waxes) by touching the antennae with glass rods smeared with these substances. Bees readily learn to distinguish between substances collected from workers of different ages, workers or eggs from the same hive, and animals with a different genetic relationship.

ANOTHER CONDITIONING PARADIGM: ANTENNAL RESPONSE CONDITIONING The coordinated movement of the antennae in response to visual stimuli, e.g. movement of a striped pattern, can be conditioned appetitively (Erber & Schildberger 1980). A strong bias was found for the direction of movement: Only ventral to dorsal movement simulating an approach flight is associated. Erber and coworkers (Erber et al. 1993) developed another paradigm in which the spatial screening movement of the antenna is conditioned. The antenna more frequently probes an area where it experiences a flat obstacle. The memory for the localization of the obstacle lasts several minutes. This paradigm is particularly suitable for the study of modulatory actions of transmitters in the bee brain (Erber & Kloppenburg 1995, Kloppenburg & Erber 1995).

GENETICS OF LEARNING Behavioral traits, including foraging behavior, have strong genetic components (Fewell & Page 1993, Moritz & Brandes 1987,
Robinson & Page 1989). Analysis of these traits is facilitated by the fact that all workers in a colony are sisters, and the male bees (drones) are haploid. Drones perform equally well in olfactory PER conditioning. Learning, as expressed in PER conditioning and color learning in freely flying bees, has a high heritable component (Bhagavan et al. 1994, Brandes 1988), and lines of good or poor learners are selected within one or two generations (Benatar et al. 1995, Brandes 1987). Improvement and reduction of learning in the selected lines is independent of the sensory modalities (olfaction, color vision) and of the learning set (PER conditioning, freely flying bees) (Brandes & Menzel 1990). A correlation analysis of the genetic effects of the nonassociative and associative components in PER conditioning reveals that lines of poor learners lack the initial high response rate indicative of US-induced sensitization (Brandes et al. 1988). In a recent study, Bhagavan et al. (1994) show that genotype influences learning more strongly than age or ethotype.

OLFACTORY CONDITIONING: NEURAL SUBSTRATES

The Olfactory Pathway

The axons of ~30,000 chemoreceptors on each antenna project to 156 glomeruli of the antennal lobe (Flanagan & Mercer 1989a), where they synapse with ~4700 local interneurons and ~1000 projection neurons. The projection neurons leave the antennal lobe in three main tracts: (a) the median antennal-glomerular tract (mAGT), which reaches the calyces of the mushroom bodies (mb) first and then the lateral protocerebrum; (b) the lateral AGT (lAGT), which innervates the lateral protocerebrum first and then the calyces; and (c) the medio-lateral AGT (mlAGT), which projects only to the lateral protocerebrum, a region of the brain where all three tracts terminate on descending neurons. A major portion of the mAGT contains acetylcholinesterase (AChE) and ACh-receptors, suggesting that the olfactory input to the mbs is partly cholinergic (Kreissl & Bicker 1989). The mb is formed by 170,000 densely packed local neurons, the Kenyon cells (Mobbs 1982, Witthöft 1967). The dendrites of the Kenyon cells receive inputs from the two projection neuron tracts in the upper part of the calyx, the lip region. Other parts of the calyx receive inputs from other modalities, the collar from visual neurons, the basal ring from visual and olfactory neurons. Output neurons connect the two lobes of the mb, a and p lobes, with its own input region, the lateral protocerebrum, the contralateral mb, and many other brain regions (Menzel et al. 1994, Rybak & Menzel 1993).

Neurons of the olfactory pathway were examined with respect to their olfactory-coding properties (Flanagan & Mercer 1989b, Gronenberg 1987, Homberg 1984, Sun et al. 1993), and a single identified mb-extrinsic neuron,
the PE1, was studied extensively in the context of nonassociative and associative learning (Mauelshagen 1993). The response of PE1 to olfactory stimuli does not change in a sensitization protocol with a compound sucrose stimulus applied to antennae and proboscis, but selectively, as a consequence of forward-pairing trials of the conditioned odor and this sucrose stimulus. A single forward pairing leads to a reduction, and multiple trials to an increase, in its CS-evoked response. Both the single- and multiple-trial effects are transient and disappear at longer (>10 min) intervals. Other forms of neural plasticity in the olfactory pathway were described by Erber (1981).

**The US Pathway**

Sucrose receptors at the antennae and proboscis project to the subesophageal ganglion and terminate in close apposition to premotor and motorneurons involved in PER (Rehder 1989). In addition, a group of ventral unpaired median (VUM) neurons receive input from the sucrose receptors. One of the neurons, VUM$_{mx1}$, was found to be sufficient to serve the US-reinforcing function when its activity follows the CS at an optimal time interval for forward PER conditioning (Hammer 1993). The axonal arborizations of VUM$_{mx1}$ converge with the olfactory (CS) pathway at three sites, the antennal lobe, the lateral protocerebral lobe, and the mb calyces (lip and basal ring). VUM$_{mx1}$ was depolarized shortly after CS presentation in order to demonstrate that it mediates reinforcement; it thus substituted for the US in a single olfactory trial. A conditioned response to the CS was found after forward pairing, but not after backward pairing, of the CS with the depolarization of VUM$_{mx1}$. The transmitter of the VUM neurons is most likely octopamine, because these neurons stain with an antibody against octopamine (Kreissl et al. 1994). Because VUM activity does not activate the reflex pathway, VUM$_{mx1}$ may specifically serve US reinforcement in olfactory PER conditioning via octopamine release; it executes this function in two neuropiles in parallel (antennal lobe, calyx of mb) (Hammer & Menzel 1995).

Knowledge of the neuron representing the US-reinforcing function in PER conditioning provides the opportunity to ask whether VUM$_{mx1}$ exerts properties that may allow the tracing of behaviorally unobservable variables, such as expectancy, attention, and certain forms of stimulus representations governing the associability of stimuli in inhibitory learning, blocking, and second-order conditioning. For example, VUM$_{mx1}$ develops a response to CS$^+$ but not to CS$^-$ in differential conditioning (Hammer 1993). The prolonged response to CS$^+$ may be a neural substrate for second-order conditioning, since it occurs in a neuron that has a reinforcing function. Other properties of VUM$_{mx1}$ have yet to be analyzed.
Localization and Dynamics of the Olfactory Memory Trace

Local cooling of selected parts of the bee brain induces retrograde amnestic effects which depend on the time interval between the single learning trial and the site of cooling (Erber et al. 1980, Menzel et al. 1974). The time course of growing resistance to amnesia induced by cooling the calyx region of the mbs resembles that for cooling the whole animal with a half-effect time interval of 3-4 min. Corresponding half-effect intervals are 1-2 min for the antennal lobes and 2-3 min for the a lobes. No amnestic effect occurs when the lateral protocerebral lobe is cooled. These results indicate that the mbs, in particular their input sites, the calyces, are essential structures for the formation of an amnesia-resistant memory trace but that other structures, such as the antennal lobes, participate at an early stage of the memory trace. The role of the mbs in insect olfactory learning is supported by the projection pattern of the VUMmx1 neuron (see above) and the finding that structural mb mutations (Heisenberg et al. 1985) and chemically ablated mbs in D. melanogaster (de Belle & Heisenberg 1994) lead to a total loss of olfactory learning, with negligible or no sensory and motor side effects.

One of the questions arising from these results is whether the mbs are the only substrate for the consolidation of associative olfactory learning. This question can be approached for the honey bee because the neural substrate of the US pathway is known, and its activity in the various neuropiles can be simulated by injecting the presumed transmitter, octopamine (OA), into these neuropiles as a substitute for the US in CS-OA pairing trials. Under these conditions, bees develop a conditioned response to the CS for both the injections into the calyx and the antennal lobe but not into the lateral protocerebrum, indicating that an associative trace is established in either of these two structures and independently from each other (Hammer & Menzel 1994). Another question relates to the problem of whether US-induced sensitization is a requirement for associative learning. Reserpine depletes biogenic amines in the bee brain, as in vertebrate brains, and causes a pronounced reduction in sensitization and conditioning (Braun & Bicker 1992, Menzel et al. 1991). Injection of OA into the brain of reserpinized bees prior to conditioning selectively restores conditioning but not sensitization (Menzel et al. 1993c), indicating that sensitization is not necessary for associative learning. A third general issue, which can be addressed using PER conditioning, relates to the problem of how the two components of memory, retrievability and memory formation, are connected. Dopamine injected into the brain blocks memory retrieval, but learning is not affected (Menzel et al. 1988). Dopamine restores the motor components of PER in reserpinized animals but not the learning-related components (Menzel et al. 1993c). Furthermore, satiated animals show neither the sucrose-released reflex nor the sucrose-induced sensitization, but olfactory...
conditioning is still possible, as indicated by the conditioned response when the animals are hungry (J Klein, M Hammer & V Steffen, personal communication). Thus retrieval and memory formation may be two separable processes in PER conditioning.

Repeated learning trials lead to amnesia resistant long-term memory (LTM) within less than a minute (Menzel & Sugawa 1986). The accelerated transfer into LTM requires associative trials and does not occur with CS-only or US-only trials. The question has been posed whether LTM in bees depends on protein synthesis, as it does in vertebrates (e.g. Squire & Davis 1975) and in long-term facilitation in Aplysia sensory neurons (Schacher et al. 1988).

Injection of cycloheximide directly into the bee brain blocks protein synthesis to a high degree (~95%), but neither learning nor LTM is reduced. This finding applies to olfactory PER conditioning (Menzel et al. 1993a, Wittstock et al. 1993) and color learning in freely flying bees (Wittstock & Menzel 1994). LTM was tested up to three days after learning. These results do not exclude the possibility that bees, too, have protein synthesis-dependent forms of LTM, but if they exist, they do not limit long-term performance in conditioned behavior tested so far. In fact, two parallel forms of LTM were found in D. melanogaster recently, one lasting about four days and insensitive to protein synthesis inhibition or cooling, and the other lasting longer than four days and sensitive to protein synthesis inhibition and cooling (Tully et al. 1994). The latter form of LTM requires de novo gene expression, probably mediated by CREB protein (Yin et al. 1994).

Thus, at the circuit level, the neural substrate of olfactory PER conditioning is characterized by two neuropiles (antennal lobe, mb) and three tracts (the m-, ml- and lAGTs). The antennal lobes and mb calyces, but not the lateral protocerebrum, appear to develop an associative memory trace. The mb calyces receive inputs from other sensory modalities, and the mbs are the substrates for intense multisensory integration at the highest level of the insect nervous system (Erber et al. 1987, Menzel et al. 1994, Mobbs 1982, Rybak & Menzel 1993). Thus these memory traces in the antennal lobes and mbs may store different aspects with respect to the CSs involved—the nonassociative and associative components and the reinforcing effects (e.g. excitatory and inhibitory conditioning).

CELLULAR AND MOLECULAR SUBSTRATES OF OLFACTORY LEARNING

A first step toward the elucidation of the cellular and molecular mechanisms of learning and memory relates to the identification of first and second transmitters involved and to the potential role of protein kinases and their substrates. The putative first transmitters were identified (CS pathway: ACh; US pathway:...
OA) and protein kinases known to play a key role in cellular plasticity were characterized in the bee (Altfelder & Müller 1991, Altfelder et al. 1991, Müller & Altfelder 1991).

Second-Messenger Pathways

ANTENNAL LOBE: MODULATION OF PROTEIN KINASE A BY THE US AND CS  Second-messenger-regulated protein kinases in the antennal lobe (AL) were analyzed to investigate whether they are affected by the US and CS. Whereas application of sucrose to an antenna causes a rapid and transient elevation of PKA activity in the AL, mechanosensory or CS stimulation of an antenna does not affect protein kinase A (PKA) activity (Hildebrandt 1994; Hildebrandt & Müller 1995a,b). Because the transient modulation of the PKA is evoked by the OA-cAMP system but not by other monoamines detected in the AL (Mercer et al. 1983) the VUM

MUSHROOM BODIES: SIGNALING CASCADES  The predominant expression of gene products of D. melanogaster memory mutants dunce and rutabaga encoding for a cAMP phosphodiesterase and a Ca^{2+}-calmodulin-dependent adenylyl cyclase in the mbs indicates the crucial role played by the cAMP cascade during learning and memory at the mb level (Davis 1993). Although how and which synaptic mechanisms are affected by the cAMP cascade is still unknown, the cAMP pathway and its targets are of the utmost importance in mechanisms of learning in D. melanogaster (Drain et al. 1991, Yin et al. 1994). Biochemical determination of kinase activity in distinct areas of the bee's mbs reveals a two- to fourfold higher activity of PKA, protein kinase C, and Ca^{2+}-calmodulin-dependent kinase, compared to other neural tissues. This, taken together with the distinct localization of still uncharacterized substrate proteins of the kinases on the input and output sides of the mb, points to a complex network of different second-messenger cascades implicated in the processing of neuronal signals in the mbs (Hildebrandt 1994; U. Müller, unpublished observations).

MODULATION OF CURRENTS IN KENYON CELLS  The intrinsic elements of the mb, the Kenyon cells, are the potential common postsynaptic sites of the CS and US pathway, and thus were examined with respect to their currents and receptors. Whole-cell patch measurements were performed on dissociated, short-term cultured Kenyon cells (Kreissl & Bicker 1992). Five voltage-de-
pendent currents were characterized: a fast inactivating TTX-sensitive Na current, a typical insect Ca current that is insensitive to co-conotoxin, a rapidly inactivating A-type K current, a charybdotoxin sensitive Ca-activated K current, and a delayed rectifier current (Schäfer et al. 1994). Application of α-amino-γ-butyric acid (GABA) evokes a picrotoxin-sensitive chloride current, while ACh activates a nonselective cation current with a high proportion of calcium. The ACh receptor turned out to be very similar to the neural vertebrate receptor (Zhang & Peltz 1990).

Transmitters, known to be involved in learning and memory (Bicker & Menzel 1989), were tested for their modulatory effects on the ACh and the A currents. Histamine, serotonin, and glutamate increases, and noradrenaline decreases, A current. The transmitters OA, tyramine, dopamine, were found to be ineffective on the A current, and serotonin reduces the ACh-mediated Ca\(^{2+}\) current reversibly (Rosenboom et al. 1994). In addition, high intracellular Ca\(^{2+}\) reduces the ACh current. Modulation of the ACh-mediated Ca\(^{2+}\) current by OA is of special interest, since OA is the putative transmitter of the US pathway, which may converge with the cholinergic CS pathway on the dendrites of the Kenyon cells.

Besides patch-clamp recordings, Ca\(^{2+}\)-fluorescence measurement on Kenyon cells reveals additional evidence of an ACh-mediated Ca\(^{2+}\) influx (Bicker & Kreissl 1994, Rosenboom et al. 1994). Moreover, the Ca\(^{2+}\) measurements are currently used to elucidate the function of transmitter evoked changes in Ca\(^{2+}\) level and its cross talk with components of other second-messenger cascades.

TOWARD AN UNDERSTANDING OF CELLULAR PATHWAYS The biochemical analysis of the coupling between transmitters, modulators, and second messengers turns out to be rather complex in the mbs, because (a) receptor subtypes exist that differ in their effects on second messengers, (b) multiple cross talk occurs between different second-messenger systems, and (c) distinct Kenyon cell subpopulations express different components of the second-messenger cascades. Initial evidence of the expression of special OA receptor types in the mbs is indicated by the distinct pharmacology of OA-binding sites in the mbs, compared to other brain areas (Erber et al. 1993). In addition to a subclass of OA receptors that stimulates adenylyl cyclase (Evans & Robb 1993), a distinct, separate G-protein coupling has been demonstrated for a cloned D. melanogaster OA-tyramine receptor (Saudou et al. 1990). While OA is more effective in the elevation of intracellular Ca\(^{2+}\) levels, tyramine is more potent in inhibition of adenylyl cyclase activity (Robb et al. 1994). Although it is not yet possible to relate receptor subtypes and their coupling with G proteins to distinct functional areas within the mbs, biochemical evidence supports the idea of different OA receptor G-protein coupling in the mb's calyces. In
contrast to the OA receptors in the AL, which activate adenylyl cyclase, OA has no direct effect on PKA activity in the mbs (Hildebrandt 1994).

No specific hypothesis for the processing of the US and CS in the mbs can yet be presented at the molecular level. Further studies need to take into account the functional differences between Kenyon cells, as indicated by biochemical and immunohistochemical findings (U Müller, unpublished observations).

Nitric Oxide and Its Potential Functional Role

Nitric oxide (NO) was recently identified as a signaling molecule in the brain of bees (Müller 1994). In the mammalian nervous system the role of the free radical gas NO as a signaling molecule is well documented (Dawson & Snyder 1994, Schuman & Madison 1994a). Apart from its other functions, NO seems to play a role in the modulation of synaptic functions like long-term depression and potentiation and animal learning and in the development and regeneration of the nervous system (Dawson & Snyder 1994, Schuman & Madison 1994a,b). In contrast to conventional transmitters that are restricted to single synapses, NO easily diffuses from its site of production through membranes to act on neighboring targets. Therefore, the spatial distribution of NO within a cellular compartment provides a potential mechanism of modulation of neuropilar volumes, limited by half-life, diffusion constant, and diffusion barriers (Breer & Shepherd 1993).

NO synthase (NOS) in the bee brain has properties similar to those described for the vertebrate NOS. NOS-expressing cells and neuropiles can be reliably identified by NADPH-diaphorase histochemistry (Müller 1994). The localization of NOS in the AL and the tip of the mb calyces, sites of CS and US convergence, suggests that the NO system plays a role in the processing of chemosensory information and possibly also in mechanisms of learning and memory. In the AL, interneurons, which have an integrative function in chemosensory signal processing (see above), are responsible for the high NOS concentration within the glomeruli. Thus, the release of NO from AL interneurons (Müller & Bicker 1994) may modulate lateral mechanisms in neighboring cells within the circuitry of the glomeruli, which contribute to sensory adaptation and habituation (Breer & Shepherd 1993). Local inhibition of the NO-cGMP system in a single AL specifically affects habituation (Hildebrandt et al. 1993, Müller & Hildebrand 1995). Repetitive chemosensory stimulation in vivo causes a gradual increase of PKA activity in the AL, mediated by NO activation of a soluble guanylate cyclase (Hildebrandt et al. 1994). Thus, in the bee, the NO-cGMP system in the AL is a component of the machinery involved in nonassociative adaptive mechanisms during chemosensory information processing, as supposed by Breer & Shepherd (1993). Recent findings indicate that inhibition of the NOS in the whole brain
interferes with components of long-term associative odor memory (U Müller 1995). Inhibition of the NOS during the CS-US pairing (three trials, intertrial interval 2-6 min) alters neither US-induced sensitization, nor acquisition of a conditioned response (CR) to the odor during conditioning, nor memory retrieval up to 12 h after conditioning. However, memory tests 24 h after conditioning reveal that no LTM is formed. Interestingly, inhibition of the NOS during a single CS-US pairing does not affect the CR tested 24 h after conditioning, indicating that LTM requires multiple learning trials (see above). The molecular target of NO action, mediating LTM, is as yet unknown. In both vertebrates and insects, a major effector of NO is the soluble guanylate cyclase (Elphick et al. 1993, Schuman & Madison 1994a). The cGMP produced via activation of the NOS can modulate different components of the cAMP cascade, e.g. it can activate PKA (Altfelder & Müller 1991), change the cAMP levels by modulating phosphodiesterases, and interact with cyclic nucleotide-regulated ion channels (Dawson & Snyder 1994, Schuman & Madison 1994a). As a result, the release of the diffusible messenger NO during CS-US pairing may modulate cAMP-mediated signaling in adjacent neurons, possibly the Kenyon cells. Furthermore, NO represents a suitable transmitter for the synchronous coupling and/or strengthening of populations of neurons synaptically connected within a confined area (Schuman & Madison 1994b) that may also be required for distinct components of memory formation. Such networks oscillate, and NO-mediated changes in oscillations seem to play a role in the neuronal processing of chemosensory information, as recently reported for the mollusc Limax maximus (Gelperin 1994). In this context it is tempting to ask whether NO is also implicated in the odor evoked oscillation in subsets of Kenyon cells. Such oscillations were found recently in the locust brain (Laurent & Naraghi 1994, Laurent & Davidowitz 1994), but whether the oscillations are related to olfactory recognition and/or olfactory learning is still unknown.

CONCLUSIONS

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. Freely flying bees in a controlled environment learn in a way that can be interpreted with reference to the ecological conditions faced by a social animal that adapts its foraging efforts to fast and unpredictable changes in the food market. Olfactory conditioning of harnessed bees 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 connectivities. Analysis of 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, neuropilar, and behavioral levels of associative learning.

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