CHAPTER

29

In Search of the Engram in the Honeybee Brain

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THE CONCEPT OF THE ENGRAM

Memories exist in multiple forms and have multiple functions. They may be categorized according to their cell-physiological substrates along a timescale defined as short-term, mid-term, and long-term memory (STM, MTM, and LTM, respectively). Ongoing neural activity serves as the storage device for STM; intracellular signaling cascades lead to MTM; and gene activation, protein synthesis, and new structures underlie LTM. The physiological substrates of these memory stages or phases can be sequential or in parallel, indicating that memory systems are highly dynamic. But what exactly is processed and stored in these different phases of memory formation? Memory is about something, namely objects, events, and relations between objects in the external world as well as internal body states such as hunger and satiety. Thus, memory stores information about the meaning of multiple signals, external and internal; in other words, it has content. Stimuli and actions are evaluated by the nervous system according to expected outcomes, and it is this loop into the future that defines the core of memory content. The ultimate goal of memory research is to uncover the neural mechanisms that allow the content of memory to be encoded, stored, processed, and retrieved. The content of memory is usually considered to be encoded as an engram or memory trace. Lashley[1] referred to the engram in the title of his famous paper and asked questions concerning the location of the engram(s) in different parts of the mammalian brain (cortex). Localization is indeed a major feature of memory, and in the mammalian brain memory localizations can be categorized according to the types of memory that are processed—for example, procedural memory (e.g., cerebellum), episodic memory (hippocampus and prefrontal cortex), and emotional memory (amygdala)—but the content of each of these memories involves many more parts of the brain. Another character of memory is content-sensitive processing.[2] Any retrieval from the memory store changes its content due to the updating process in working memory, a process referred to as 'reconsolidation.' It is this updating process that may reveal rules underlying generalization, categorization, and implicit (and explicit in humans) forms of abstraction. However, both localization and content-specific processing tell us little about the mechanisms of how content is encoded and stored in the nervous system, although knowledge of both aspects of the engram is requirements for hypothesis-driven research.

Cognitive psychology has struggled with the question of whether the engram or memory trace 'exists' if it is not retrieved. "Where is the memory trace when we are not remembering? ... The hunt for the engram (the physical manifestation of the memory trace that is independent of the operations needed to recover it) may prove to be fruitless as the hunting of the Snark".[3] Indeed, the engram is not yet a memory if it is not activated, but it is the necessary physical condition for memory to emerge through the readout process in the nervous system. In this sense, the engram, together with the neural processes of activating it and combining it with the information provided by the retrieval process, leads to memory. The informational content of the engram is therefore rather elusive,[4] and we characterize our efforts better by saying that we aim to uncover necessary physical components that we hope will define (at least to some extent) the informational content of the trace leading to the engram. These attempts will be very limited because in reading these physical components as separate entities, the emerging properties from the interaction of multiple components necessary to

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convert the isolated traces to the engram will be extremely difficult to discover.

Memory content requires ubiquitous molecules and chains of cellular reaction cascades but is certainly not encoded and stored in such elemental processes. Rather, memory content is expected to result from the spatial distribution of learning-dependent changes of neural activity and synaptic transmission resulting in reorganized neural nets. Such a view follows Ramon y Cajal's[5] view that the engram is expressed in novel brain structures. Addressing "Hebb's dream," as Nicolelis and co-workers[6] called it, the uncovering of the engram thus requires capturing the changing structures and the dynamic processes hidden in learning-dependent changes of whole neural nets. Ideally, one would like to elucidate all these components of neural nets with subcellular resolution, a demanding task for higher organisms but possibly not out of reach for invertebrate brains.

In this chapter, I discuss attempts to follow this line of argument by characterizing the olfactory engram in the honeybee brain that develops in the course of odor/reward learning. Early in my research career, I would have liked to address this question for visual (color) learning in bees because my major interest was studying behavior in bees. Because bees do not learn colors well (or do not reveal their visual learning of the proboscis response) under conditions that allow brain recordings, I needed to shift to olfaction, a perceptual modality that makes stimulus quantification much more difficult. Furthermore, at the time these efforts started (1985), very little was known about primary sensory coding of odors in the insect brain (and in other brains). In hindsight, this forced detour was favorable because it led us to think about methods that allow simultaneous measurements of neural excitation in neural nets under conditions in which an animal learns, encodes, stores, retrieves, and prepares for actions on the basis of a memory trace.

THE OLFACTORY LEARNING PARADIGM

Learning takes many forms and plays an essential role in honeybee behavior. Latent (observational) and associative learning (operant and classical conditioning) interact in natural behavior. On their first flights out of the colony, honeybees explore the environment and learn the spatial relations of landscape structures relative to their hive location within a reference system, the sun compass, by relating the sun azimuth time function to extended landmarks.[7] They attend to waggle dances, receive the information about distance and direction of the outbound flight toward the indicated location, and apply this information within the frame of the experienced landscape. Olfactory cues sensed during dance recruitment are learned and searched for when localizing the communicated place.[8] At a feeding site, they associate the signals (odors, colors, shape, manipulatory components, spatial location relative to nearby landmarks, and time of the day) with the quality and quantity of reward, nectar and pollen. Multiple visits to the feeding site allow them to extract features such as the change of reward quantity over time[9] and to the reliable components of the signals such as symmetry[10,11] or the consistent components within variable odor mixtures.[11]

The memory traces resulting from such rich forms of learning store not just the stimulus-response associations but also derived representations characterizing the where, when, and what components of evaluated experiences (see Chapter 3 for further arguments in favor of a cognitive interpretation of learned representations in honeybees). It is also important to recognize the dynamic structure of the memory trace. Four memory stages (in addition to a sensory memory in the seconds range) can be distinguished depending on the respective time courses controlling behavior, sensitivity to interference, and the molecular cascades involved,[12] resembling the general structure of memory dynamics in other invertebrates and mammals.[13] Consolidation from STM to MTM requires ongoing neural activity in the minutes range and activation of protein kinase M (PKM; mediated by the proteolytic cleavage of PKC in the hours range,[14] whereas consolidation of STM to LTM requires the activity of the cAMP-dependent PKA in the antennal lobes.[15] Early long-term memory (eLTM) depends on translation (1 to 2 days after conditioning) and late long-term memory (lLTM) on transcription and translation (>3 days after conditioning). Both forms of LTM develop in parallel.[16] LTM lasts for the lifetime of a bee, which may be more than 6 months in overwintering animals.[17] Formation of LTM requires multiple (three or more) learning trials, whereas STM and MTM can be formed after one learning trial.[18]

Control of ongoing behavior at any given moment is supervised by working memory, a "limited capacity system for maintaining and manipulating information . . . allowing for complex and flexible cognition".[19] The span of working memory in freely behaving honeybees has been uncovered by several experimental procedures. Short-term working memory (in the seconds range) was observed in maze learning,[20] matching-to-sample tests,[21] and serial learning tasks.[22] Longer term working memory (in the range of several minutes) was reported in tests in which the

quantitative reward conditions were made contingent on the animals' own behavior.[23] Very long working memory (in the range of hours to days) was found in tests that involved learning of incentive gradients.[9] In the latter two cases, working memory is characterized by the retrieval of context-specific memory that is used to evaluate and update current experience. Directed attention, a characteristic component of working memory, has yet to be addressed in honeybee research.

The search for the neural correlates of memory calls for experimental conditions in which a bee learns to associate a stimulus with reinforcement and forms a lasting memory trace. Olfactory reward conditioning of the proboscis extension response (PER) is a robust paradigm that allows combining behavioral with neural studies. Foraging bees are collected at the hive entrance, cooled, and harnessed in a tube so that the antennae and mouthparts are freely moving but the legs, wings, and abdomen are encased. The conditioned stimulus (CS+; odor, mechanical stimuli, CO_2, humidity, and temperature) is applied to the antennae and subsequently rewarded with sucrose reward (unconditioned stimulus (US). Hungry bees extend the proboscis when the sucrose receptors on the antennae are stimulated. The proboscis is then allowed to lick sucrose solution for a few seconds. The optimal time interval between onset of CS+ and US is 2–4 sec. Bees acquire the conditioned response to CS+ within a few trials and retain it after several training trials as long as they can be kept alive under these conditions (up to 1 week if fed to satiation every evening). Backward conditioning (first US and then CS+; optimal interval, 20 sec[24]) leads to inhibitory learning, as indicated by resistance to subsequent acquisition. Multiple conditioning procedures have been tested, including trial spacing effects, second-order conditioning, conditioned inhibition, extinction learning and spontaneous recovery from extinction, compound processing, and occasion setting (25; see also Chapter 33). Performance values are usually group-average learning curves; however, such group effects do not adequately represent the behavior of individual animals, an important finding because correlations between behavior and neural correlates need to be established on the basis of individuals. Individual behavior is characterized by a rapid and stable acquisition of the conditioned response (CR), as well as by a rapid and stable cessation of the CR following unrewarded stimuli (extinction). Two processes interact during classical conditioning—a gradual and an all-or-none learning process. Thus, individual behavior is a meaningful predictor for the internal state of a honeybee irrespective of the group-average behavioral performance.[26]

THE OLFACTORY PATHWAY IN THE BEE BRAIN AND POTENTIAL LOCATIONS OF THE ENGRAM

Sensory neurons expressing the same olfactory receptor converge on aggregates of glomerular neuropil structures in the antennal lobe (AL),where they synapse onto local interneurons and projection neurons (PNs) that connect the mushroom body (MB) and the lateral horn (LH) via two tracts, the median and later antennoprotocerebralis tracts (mAPT and lAPT, respectively) (Figure 29.1). In both vertebrates and invertebrates, the combinatorial pattern of neural activity in the glomeruli is odor specific. In insects, the PNs transmit this pattern to the next synapse in the calyces of the MB. Here, neural excitation diverges from approximately 800 PNs to a large number (>100,000) of MB intrinsic neurons, the Kenyon cells (KCs). Each of the more than 1 million presynaptic boutons of the PNs comprise a microcircuit composed of approximately 10 postsynaptic sites of KCs and both pre- and postsynaptic sites of putative inhibitory neurons of the protocerebral-calycal tract (PCT). KCs may collect their input either exclusively in one of the three calycal compartments (lip receiving input from olfactory PNs, collar receiving visual input, basal ring receiving mixed input from olfactory and mechanosensory input; Kenyon cell type I (KC I)) or across these calycal compartments and then project their axons to both lobes of

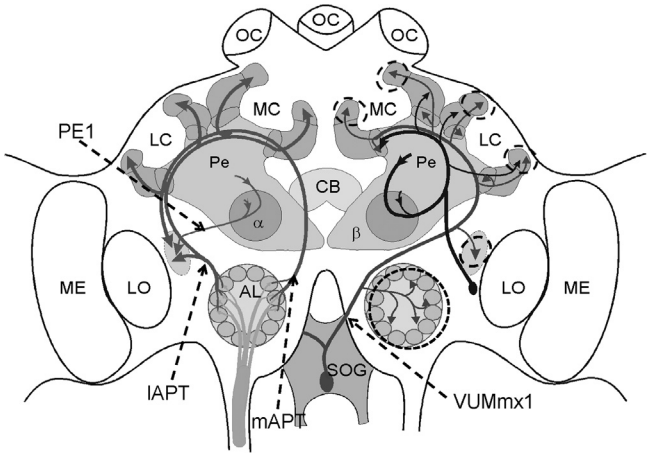

FIGURE 29.1 **The olfactory pathway (left half of the brain) and reward pathway (right half of the brain) in the honeybee brain.** The convergence sites between olfactory and reward pathways are marked by dotted circles in the right side of the brain. α and β, alpha and beta lobes of the mushroom body; AL, antennal lobe; CB, central body; LC and MC, lateral and median calyx of the mushroom body; LO, lobula (third visual ganglion); ME, medulla (second visual ganglion); OC, ocelli; Pe, peduncle of the mushroom body; SOG, subesophageal ganglion. The dashed arrows point to lAPT and mAPT (lateral and median antennoglomerular tract, respectively), the PE1 neuron, and the VUMmx1 neuron.

the MB (α and β lobes; Kenyon cell type II (KC II)). The axons of both types form collaterals halfway along the peduncle and project to the α and β lobes. KC I project one collateral to the dorsal α lobe and the other to the caudal part of the β lobe. KC II project one to the ventral part of the α lobe and the other to the proximal part of the β lobe.[27] The large number of KCs converge on a rather small number (~400) of MB extrinsic neurons (ENs), leaving the α lobe at three prominent exit points—the lateral, ventral, and ventromedian exit points.[28] These ENs are divided into seven subgroups (A1–A7) depending on the localization of their somata. Most of them are postsynaptic to KCs, as judged by their spiny-like structure and in some cases by electron microscopic evidence, but post- and presynaptic structures are known to occur in close vicinity. ENs project to many parts of the brain—some of them (e.g., the identified neurons PE1 and the A4 neurons) to different subregions of the LH, where they converge directly or indirectly with collaterals of mAPT and lAPT. Other ENs (A3 neurons) project via a recurrent pathway back to the calyx of the same MB along the GABA-immunoreactive (GABA-ir) PCT. Multiple ENs connect the ring neuropil around the α lobe (A1, A2, A4, and A7) with the MB on the ipsi- or contralateral side (A6 and A7) or with other protocerebral areas on the ipsi- or contralateral side (A4, A5, and A7). A single neuron has been identified that appears to project back to the ipsilateral AL (the AL1). Dendrites of ENs are often restricted within the α lobe to one of the horizontal bands, suggesting that they receive sensory modality-specific input via KCs. Others distribute their dendrites across the banded structure of the α and β lobes. The structural diversity of ENs reflects a multiplicity of functions concerning the readout from the MB and the information flow to other parts of the brain.

Neurons containing the neuromodulators octopamine (OA) and dopamine are related to the reinforcing functions during conditioning both in *Drosophila* (see Chapters 5 and 27) and in the bee. One OA immunoreactive neuron, the VUMmx1, was identified in a reward substitution experiment to be sufficient for the reward function of sucrose in olfactory conditioning in the bee.[29] VUMmx1 receives its input in the subesophageal ganglion and converges with the olfactory pathway at three pairs of symmetrical sites—the ALs, the LHs, and the lip regions of the MB calyces, respectively (Figure 29.1). Thus, it has been hypothesized that these convergence sites may constitute localizations of the olfactory engram as it develops in reward learning,[30] and therefore recordings of learning-related neural plasticity focused on the neurons and their synaptic connections so far on two of these three sites (AL and MB calyx). A functional MB was previously found to be required for the consolidation of olfactory STM into MTM.[31] The VUMmx1 neuron responds to sucrose and to many other stimuli. In the course of conditioning, it enhances its response to the forward paired conditioned olfactory stimulus (CS+) and reduces its response to the backward paired stimulus (CS−) (see Figure 29.10). Interestingly, regarding the notion of expectation and anticipation, the octopaminergic neuron VUMmx1 exhibits activity reflecting the animal's expectation: It responds to unexpected sucrose presentations but not to expected ones.[29] Although there is evidence in vertebrates of how this reduction in the error signal may be implemented biologically, such evidence is still lacking in invertebrates.

THE ANTENNAL LOBE

The AL of the honeybee is believed to constitute a component of a distributed network storing olfactory information, but evidence is controversial. As noted previously, convergence of the olfactory and reward pathway in the AL suggests a memory trace to be formed in the AL. Indeed, substituting reward in olfactory PER conditioning by local injection of octopamine (the putative transmitter of VUMmx1) into the AL leads to learning of the forward but not the backward paired odor.[32] Accordingly, blocking octopamine receptors in the AL with RNAi reduces olfactory learning.[33] Additional arguments in favor of a memory trace in the AL concern (1) the role of the AL in memory consolidation and (2) neural correlates of a memory trace. First, memory consolidation induced by a single learning trial was found to be blocked if the AL is cooled during the minute following the trial, whereas cooling even immediately after the last of multiple learning trials does not impair memory consolidation, suggesting that the AL possibly in connection with other brain parts stores a short-lasting memory trace necessary for consolidation.[31] Local uncaging of cAMP in the AL (cAMP promotes the transfer from STM to LTM in bees) shifts STM to LTM when it is uncaged soon after a single learning trial.[15] So far, it has not been possible to test directly whether a more permanent memory trace is stored in the AL because blocking neural activity in the AL during retrieval tests interferes with the processing of olfactory coding. Second, neural correlates of olfactory learning were collected with two methods—Ca^{2+} imaging of glomerular activity and extracellular recordings from PNs. In the first case, the Ca^{2+} signals came either predominantly from the presynaptic terminals of olfactory receptor neurons (and possibly also from glia cells) in the glomeruli or from the postsynaptic elements, the PNs. Presynaptic signals increased for

the CS+ odor.[34] Controversial data exist for associative plasticity in PNs. Peele and co-workers[35] found no consistent changes for CS+ or CS− during differential PER conditioning, whereas Weidert and co-workers[36] did. These inconsistencies may be resolved on the basis of the data from multiunit recordings of PNs. In line with the interpretation that PNs undergo associative change is the finding by Fernandez and co-workers[37] that binary mixtures of odors are coded more differently for learned odors and this effect correlates with changes of neural responses as seen in Ca^{2+}-imaging. Rath and co-workers[38] reported that PNs undergo associative plasticity in differential PER conditioning depending on their response level to the respective CS+ and CS− before conditioning: Glomeruli responding to CS+ before conditioning enhanced their CS+ responses, those that responded to CS− did not change their responses, and those that responded before both CS+ and CS− either reduced or enhanced their respective odor responses depending on the strength of their responses (weak responses were enhanced, and strong responses were reduced). The model derived from these studies assumes two types of plastic synapses in the glomeruli: (1) synapses between olfactory receptor neurons and PNs and (2) synapses between olfactory receptor neurons and local interneurons. Taken together, these results indicate that odor learning improves spatial representations of the learned odors and facilitates their discrimination—forms of specified memory traces that contribute important components to corresponding memory traces stored somewhere else.

Multiunit extracellular recordings from PNs documented both increases and decreases in rate changes to the reinforced (CS+) and the specifically not reinforced (CS−) odor (Figure 29.2),[39] but it is unknown how these effects relate to the differential associative changes seen in Ca^{2+} imaging. If such associatively up- and downregulated PNs receive their inputs within the same glomerulus (e.g., for the CS+), it is not surprising that the overall associative changes seen by Ca^{2+} imaging of whole glomeruli may cancel each other out. A model implementing these findings and making specific assumptions about spike timing-dependent plasticity induced by activity of the reward neuron VUMmx1 on the connection between local interneurons and PN predicts asymmetric changes of PN responses to the rewarded neuron.[36]

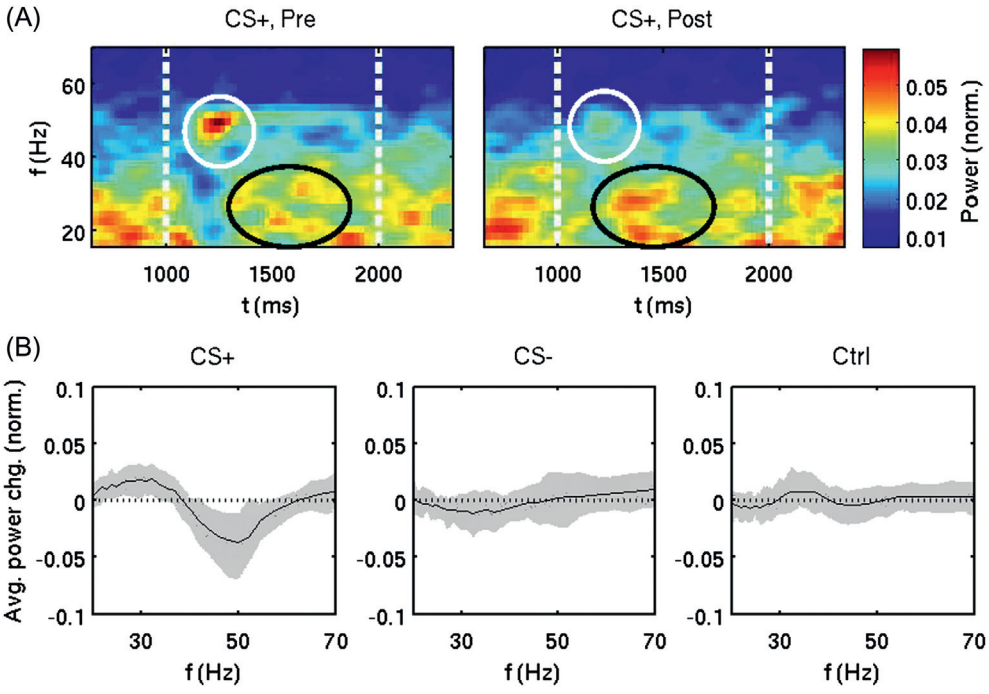

FIGURE 29.2 **Changes in LFP power of projection neurons in the course of differential conditioning as recorded by multiple extracellular electrodes.** (A) Time-resolved power spectra for CS+ tests before (left; Pre) and after (right; Post) differential conditioning averaged across three test trials for all animals. Dashed white lines indicate stimulus onset and offset; power is indicated in color scale. The white circles indicate a decrease of power in the high-frequency band, and the dark circles indicate an increase in the low-frequency band. (B) Average power change during the on-response resolved by individual frequency bands for CS+ (left), CS− (middle), and a control odor, Ctrl (right). Before averaging across animals, the differences between power before and after conditioning were calculated. Error bars (±2.5%) were obtained using 1000 bias-corrected standard bootstraps. Source: *After Denker* et al.[39]

The high temporal resolution of spike recordings allows for the analysis of the ensemble activity using odor-induced local field potentials (LFPs) and their relation to single-unit activity. The largest learning-related difference was found for CS+. LFP power increases for CS+ in the 15- to 40-Hz frequency band and decreases for frequencies higher than 45 Hz[39] (Figure 29.2). This learning-related power change correlates with the size of the neuronal ensemble that is phase-locked to the particular frequency: After learning, less units are entrained to the higher frequency band, and more units are entrained to the lower band. These results reflect associative plasticity in the AL resulting from a restructured odor coding network.

The memory trace in the AL as seen by opto- and electrophysiological recordings results from multiple training trials, suggesting that it represents a lasting trace. It optimizes primary odor coding both at the spatial and at the temporal domain. It is unknown whether other neuropils or neural tracks (e.g., feedback neurons from the MB) are required for its formation and readout and whether it contains information about the specifically learned odors. To test for this, it will be necessary to manipulate selectively the contribution of neural subsets within the AL separately for learning, consolidation, and retrieval.

INTRINSIC NEURONS OF THE MUSHROOM BODY: KENYON CELLS

MBs are expected to house the engram of insects. In 1896, Kenyon[40] stated the following:

> Ever since Dujardin[41] discovered the mushroom bodies and pointed out the relation between their size and the development of insect intelligence, nearly every writer on the subject of the hexapod brain who has referred to the matter of intelligence has recognized the fact. (p. 161)

However, even with the brilliant work in *Drosophila*, direct evidence is rather scarce. A first hint in favor of the idea that the MB is involved in memory storage came from the finding that the time course of retrograde amnesia induced by cooling the honeybee calyces matches the time course of cooling the whole animal.[31] Olfactory memory is expected to be located in the lips of the calyces because they comprise the second-order convergence sites of the olfactory pathway with the reward pathway, suggesting associative processing at the MB input. PNs are presynaptic to KCs in discrete microcircuits, the microglomeruli, composed of one large presynaptic bouton of PNs, 6–12 postsynaptic KC spines, and usually one GABA-ir profile, the presynaptic site of A3-v neurons of the PCT, and frequently a profile with dense core vesicles most likely from the OA-ir VUMmx1 neuron (Figure 29.3).

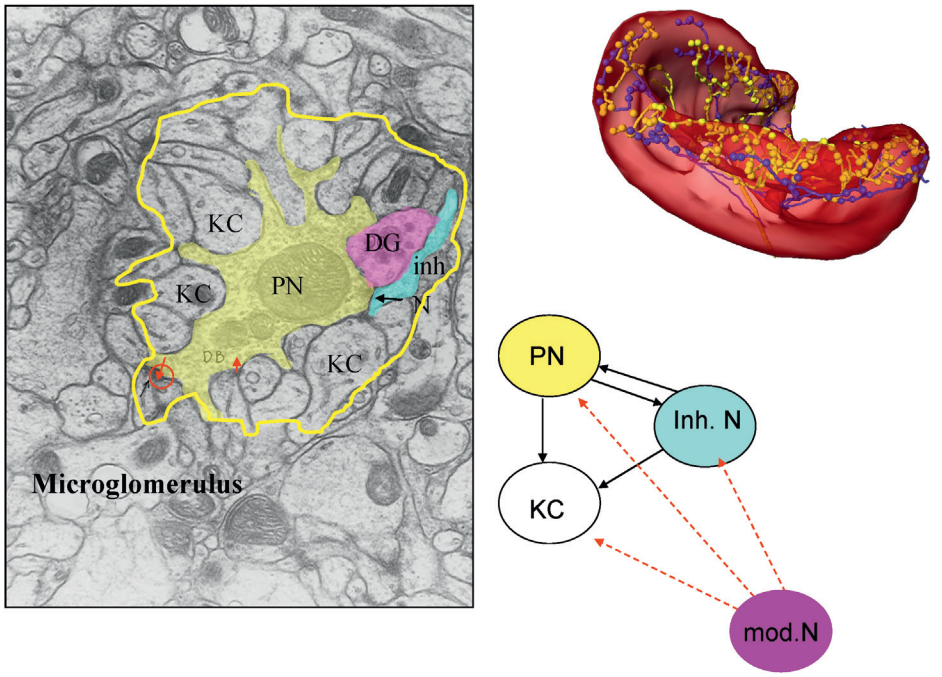

FIGURE 29.3 **Synaptic organization of the microglomerulus in the lip region of the MB.** (Top right) The terminals of two projection neurons (one in yellow and one in blue) in the lip region of the calyx with their multiple presynaptic swellings (boutons). (Left) Electron microscopic view: The large presynaptic bouton of a projection neuron (PN; surrounded by a yellow line) comprises the center of the microcircuit. It is presynaptic to multiple spines of Kenyon cells (KC) and postsynaptic to GABA-ir profiles (inh. N; blue) of the A3-v neurons. The bouton also receives input from profiles with dense core vesicles (DG; pink) interpreted to represent presynaptic sites of the reward pathway (VUMmx1). (Bottom right) The schematic representation of the microcircuit indicates directions of synaptic contacts and assumes modulatory input (mod. N) to all three partners of the circuit. Source: *After Ganeshina* et al.[42]

The density of these microglomeruli depends on the age and experience of the animals and increases during protein synthesis-dependent consolidation into olfactory LTM.[43] This latter finding was interpreted to document a structural correlate of the olfactory memory trace based on the growth of new synapses—an intriguing interpretation that will become even more convincing if it becomes possible to document stimulus-specific changes of microglomerulus patterns.

KCs feature a sparse odor code in a twofold manner: An odor activates a small proportion of highly odor-specific KCs, and in contrast to the presynaptic PNs, KCs respond with brief and phasic responses often combined with off-responses,[44] corroborating findings in the locust.[45] Stimuli of different modalities induce qualitatively similar responses, activating small subsets of KCs. Theoretically, temporal and population sparseness makes KCs potentially well suited as a memory store because the organization of the calyx can be conceptualized as an associative matrix comparable to the network of the hippocampus or cortex.[46] The memory trace as stored in such an associative matrix is characterized by features such as partial overlap between closely related traces, an optimal number of changes per trace (1–5% of the total number of synaptic contacts), and the ability to reconstruct the full pattern even if only part of the trace is activated.

Ca^{2+} imaging of the KC spines in the lip region of the calyx allowed the elucidation of learning-related plasticity of the matrix-like circuit of the MB.[47] For the first time, it was possible to reconstruct the spatial distribution of multiple changes in a neural net with a large range of partners (Figures 29.4A and 29.4B). Stimulus repetition leads to depressed responses in KCs, a form of nonassociative plasticity that is counteracted for the CS+ but not for the CS− in differential conditioning. This suggests that meaningless repetition of stimuli leads to depression, and meaningful repetition of stimuli (as indicated by the activation of the reward pathway) compensates depression possibly by selectively facilitating neural responses. Most important, KCs are either specifically recruited or eliminated from responding during odor learning, leading to a change of odor-induced activity pattern in KCs (Figure 29.4). Gain of activity (recruitment) was more often observed for the CS+ and loss of function (elimination) more frequent for the CS−, but both changes occur for both stimuli. Unchanged KC activity in the course of odor learning (shown in white in Figure 29.4A and in yellow in Figure 29.4B) are rare,

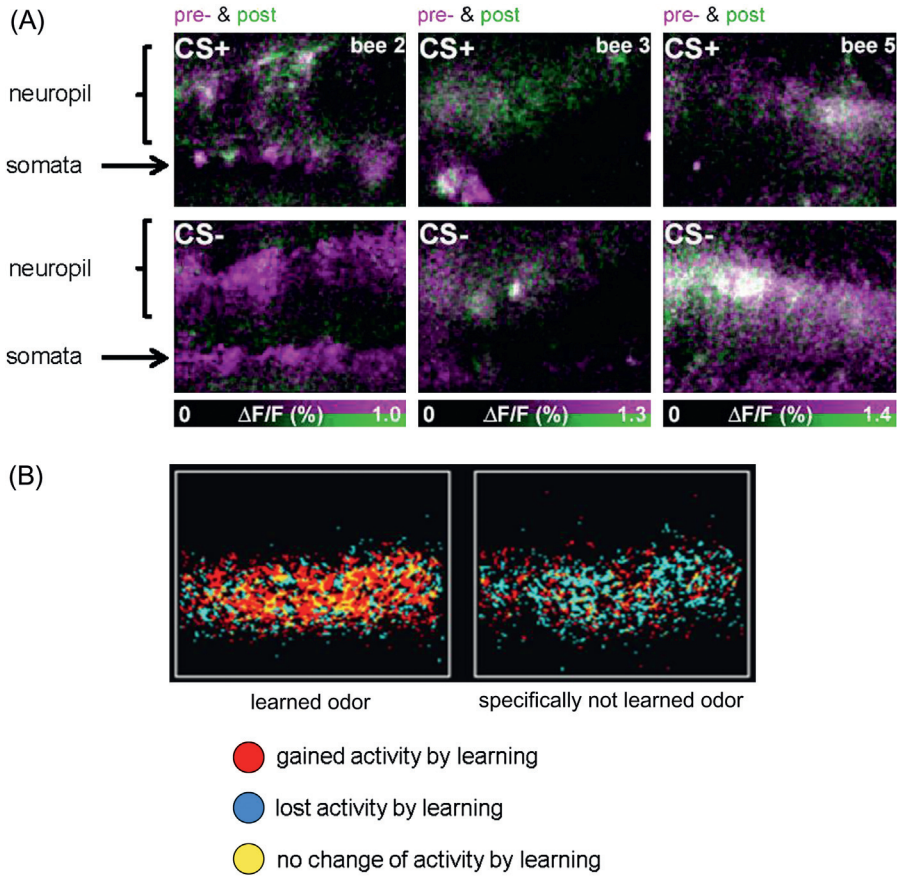

FIGURE 29.4 **Two examples of color-coded changes of KC activation patterns during differential odor learning.** (A) Response changes in three animals for CS+ and CS−. Ca^{2+} activity pattern imaged before differential PER conditioning (pre) is given in magenta, and that after conditioning (post) is shown in green separately for the CS+ (top) and the CS− (bottom). The imaged region of the MB lip shows both the somata of the clawed KCs and their synaptic neuropil. Somata and neuropil whose activity does not change during conditioning appear in white, those active only before conditioning in magenta, and those active only after conditioning in green. Odor learning leads to recruitment (green), loss of activity (magenta), and no changes (white). Bee 2 (left) is representative for the population of tested animals because it shows recruitment of activity predominantly for the CS+ and loss of activity for the CS−. (B) Example of a fourth animal in which gained activity in the course of differential conditioning is expressed in red, lost activity in blue, and no changes of activity in yellow. Sources: *Panel A after Szyszka* et al.[47]*; panel B courtesy of P. Szyszka.*

indicating that learning leads to a drastic rearrangement of odor representation in the MB input. Because odors activate primarily non-overlapping KC ensembles, the parallel representations of multiple odor traces allow for an effective and robust memory trace. In the future, it will be necessary to compare patterns of changes for odors generalized more or less. Furthermore, it will be necessary to show that multimodal stimuli lead to a more precise KC activity pattern rather than to a higher number of activated KCs.

In addition to these changes in activity patterns, KCs also undergo dynamic changes. Before conditioning, their odor responses are short, even during long-lasting odor stimulation.[43] During odor−sucrose pairing, the odor-activated KCs become reactivated, leading to coincident activity in odor coding and reward coding neurons, a possible mechanism for delayed and trace conditioning.[48] The picture emerging from Ca^{2+} imaging studies assumes an intracellular trace for the CS+, possibly in the form of a lasting increase in Ca^{2+}[49] that is associatively paired with a delayed OA input from VUMmx1 leading to an enhancement of KC activity. A reduced response to CS+ in KCs may result from a similar mechanism in inhibitory inputs to KCs—for example, via A3 neurons of the PCT, a mechanism suggested by the close apposition of OA-ir profiles and GABA-ir profiles in microglomeruli of the calyx lip (Figure 29.3). Taken together, it is conceivable that the olfactory engram in the MB lip comprises a combinatorial pattern of predominantly enhanced synaptic transmission to KCs but also reduced transmission, leading to the conclusion that KCs store a memory trace in stimulus-specific sparse activation patterns.

As noted previously, KC I receive their input selectively via small dendritic fields from lip, collar, or basal ring and project one axon collateral to the dorsal half of the α lobe and the other to the caudal part of the β lobe. KC II, to which the imaged clawed KCs belong, collect input across the calyx, thus receiving input from multiple sensory modalities via elaborate and clawed dendritic fields, and project one of their axons to the ventral part of the α lobe and the other to the proximal part of the β lobe. KC II converge on a small number of MB ENs, whereas KC I serve more ENs. One would expect the two types of KCs to process high-order sensory input and value signals differently, possibly leading to two parallel coding, storing, and retrieval schemes within the MB—a concept of high relevance in *Drosophila* MB function[50] (see Chapter 27 for further discussion of controversial data). Unfortunately, electrophysiological recordings from KCs of either type have been unsuccessful so far despite intensive efforts, and imaging experiments have not been performed in KC I. It will be an important task for the future to unravel the specific coding schemes in the two KC types as well as elucidate the potentially different roles of the median and lateral calyces with their KC projections to the inner and outer part of the lobes, respectively.

Although the MB in bees is large in comparison to the whole brain, its total volume ($25 \times 10^6\ \mu m^3$[51]) is small given the high number of densely packed KCs. Witthöft's[52] estimate of 170,000 KCs needs revision to a lower number, but even 100,000−150,000 KCs as derived from a comparison between the volume of single KCs and total MB volume (see Chapter 4) is a very high number. Obviously, MB intrinsic circuitry is designed to take advantage of small neuron size (less material, lower energy consumption) and particularly effective interneuron cross talk via short connections. It thus can be concluded that the miniaturized MB circuitry pushes information processing capacity (IPC) to an upper limit with the lowest possible volume of neural tissue, highest efficiency of use of material and metabolic energy, and shortest interneuron connections. However, axon diameters well below 0.5 μm cause problems of reliable transfer of action potentials because reduced numbers of ion channels in such small membrane areas lead to a decline in signal-to-noise ratio.[53] One important component in optimization of IPC in the MB may be related to the low spiking activity in KCs, a phenomenon well documented in the MB of locusts[45] but only indirectly assumed in the honeybee MB. IPC, rather than absolute or relative brain volumes, is considered to be the major determining factor in brain−intelligence relations.[54] The MB appears to optimize this factor by its dense packing of KCs. It will be exciting in future work to unravel the physiological and anatomical measures of IPC in KCs.

EXTRINSIC NEURONS OF THE MUSHROOM BODY

The large number of densely packed KCs in the MB converge on a rather small number (a few hundred) of MB ENs in the three output regions of the MB—the peduncle and the α and β lobes. Rybak and Menzel[28] characterized eight different groups of ENs (counting A3-v and A3-d as different groups, pooling A1 and A2 in one group, and counting PE1 as a neuron different from all other ENs) (Figure 29.5). Most of these groups contain approximately 70 neurons as judged by the number of somata (one exception: A5 comprises only 4 neurons). Four main dendritic target areas were found: (1) the ring neuropil of the α lobe to which all α lobe ENs project at least with parts of their dendrites, (2) the LH (A4 and Pe1) and optical tubercle (A5 and A7), (3) the contralateral protocerebrum

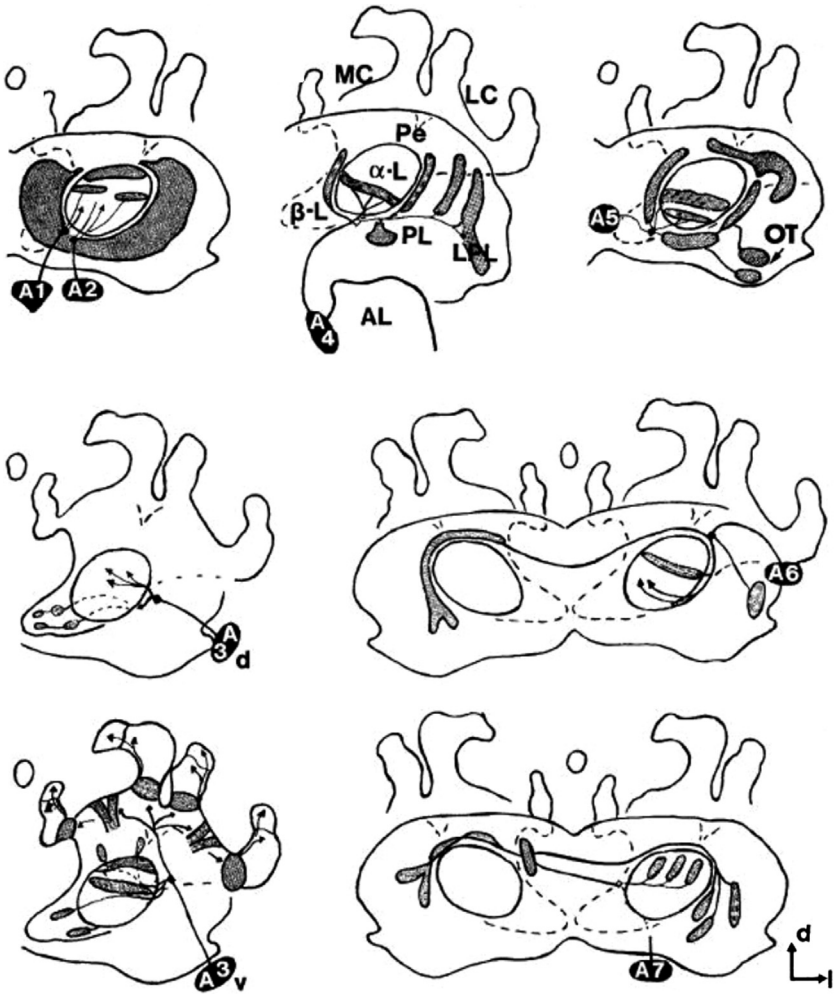

FIGURE 29.5 Schematic depiction of the α lobe ENs showing their respective clusters of somata (black circles with numbers A1–A7) and their dendritic branching areas. Source: *After Rybak and Menzel*.[28]

(A6 and A7), and (4) the feedback neurons to the calyx (A3). The multiplicity of connections established by these ENs makes it very likely that each group serves a different function. To date, these differences have not been able to be interpreted because we are ignorant about the functional characteristics of many of the target areas. In particular, we do not know the functional properties of the ring neuropil around the α lobe and the various subregions of the unstructured lateral protocerebrum. In any case, this structural multiplicity between EN groups and the number of neurons per group suggests forms of combinatorial coding of neural processing categories that are defined by the respective input and output regions. What are these categories?

Because many of these ENs receive input across the modality-specific regions of the MB, it is not surprising that they respond to a large range of sensory stimuli, indicating a different coding scheme than the highly specific combinatorial sensory code at the input of the MB. One large EN, the PE1, offers the unique possibility to repeatedly record from the same identified neuron during olfactory PER conditioning. The PE1 was found to reduce its responses to the learned odor[55] (Figure 29.6). This unique neuron receives excitatory input across the whole peduncle of the MB from KCs, also indicated by its multimodal sensitivity,[55] and inhibitory input presumably from GABA-ir A3 neurons of the PCT.[56] These latter neurons develop associative plasticity, and therefore it was tentatively concluded that enhanced inhibition via A3 neurons constitutes learned response reduction in PE1. However, PE1 also possesses intrinsic associative plasticity because KC excitation paired with PE1 depolarization induces long-term potentiation (LTP) in PE1 (Figure 29.6D).[57] It is possible that as yet unknown modulatory processes regulate transitions between LTP and long-term depression (LTD) in PE1 similar to what is known from principal cells in the mammalian cortex and hippocampus,[58] leading to associative response reduction via LTD under conditions of behavioral learning and to response

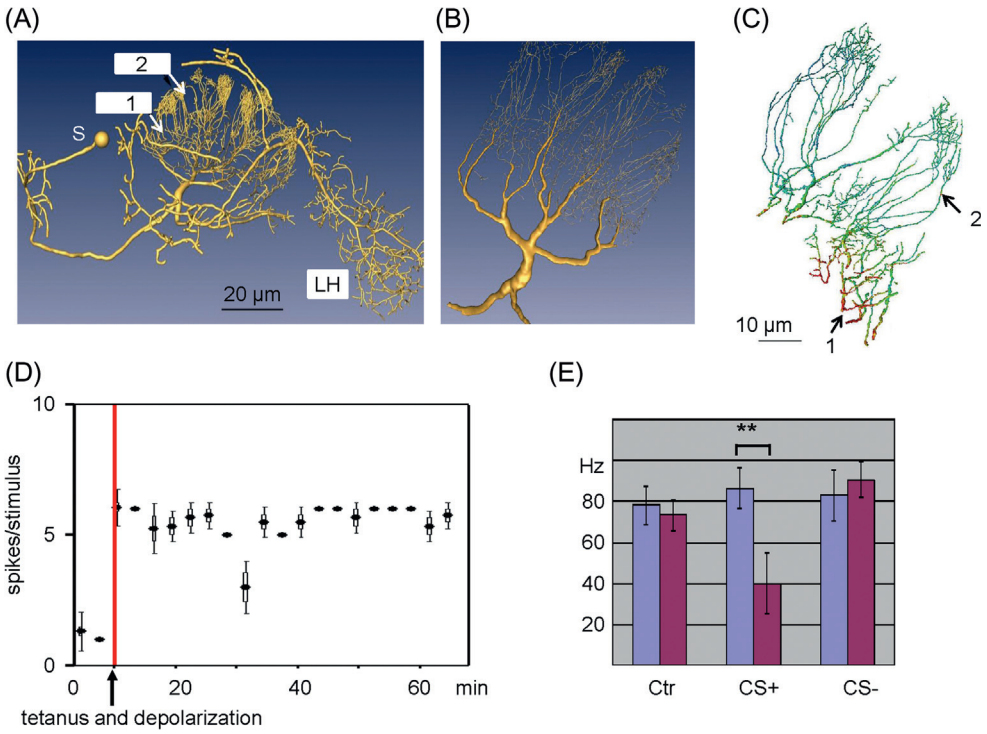

FIGURE 29.6 **Structure and plasticity of the identified MB extrinsic neuron PE1.** (A) The whole neuron with its soma (S), the dense dendrites in the MB peduncle, and the two axons—one (the neurite) leading to the soma and the other projecting to the lateral horn (LH). 1 and 2 mark two domains of the dendritic tree. (B) A higher resolution view of the dendritic tree, with the thick integrating segment and the branch point of the two axons. (C) Part of the dendritic tree (green) together with close attachments of GABA-ir profiles (red) from A3 neurons. These close attachments are found predominantly in domain 1. (D) Pairing of tetanic stimulation of KCs (arrow, 1 sec of 100 Hz) together with intracellular depolarization of PE1 leads to long-lasting enhancement of synaptic transmission (associative LTP) as seen in the number of spikes induced by each test stimulus. (E) Differential odor conditioning leads to selective reduction of CS+-induced activity. Blue bars indicate responses before and red bars indicate responses after conditioning. Ctr is a control odor. **Significant difference between the responses before and after conditioning. Source: *Panels C and E after Okada* et al.[56]; *panel D after Menzel and Manz.*[57]

enhancement via LTP under conditions of tetanic KC stimulation as used in the study by Menzel and Manz.[57] In such a scenario, associative response reduction to the CS+ would not reflect enhanced inhibition via A3 neurons but would represent an additional PE1-specific associative mechanism. Associative LTP and LTD in PE1 could also reflect spike timing-dependent plasticity (STDP), leading to either enhancement or reduction of synaptic efficiency, depending on the precise timing of spikes from KCs—a mechanism modeled for associative plasticity in the antennal lobe and reported for ENs in the locust.[59] However, STDP has yet to be proven to be related to behavioral learning in an insect.

The recorded properties of the MB from stimulus specificity to value-based responses are also demonstrated by other ENs recorded at the α exit close to the PE1.[60] Most of these neurons respond to odors with a broad chemo-profile and multiple other stimuli (visual and mechanosensory) including the sucrose reward. The sucrose responsiveness is likely to result from input of sucrose-responsive KCs that receive gustatory input via a specific ascending tract.[61] The kind of odor learning-induced plasticity in ENs varies considerably. Figure 29.7 shows four examples. Most ENs develop a response enhancement to CS+. Some of these neurons responded initially only to the US and after conditioning only to the CS+ (Figure 29.7A). Other ENs changed from excitatory CS+ responses to transient CS+ inhibitory responses (Figure 29.7C). Approximately half of the ENs recorded by Strube-Bloss *et al.*[60] changed their responses to the reinforced stimuli. Interestingly, most changed their responses not during the acquisition process but, rather, after a consolidation phase of a few hours. Two kinds of changes were observed—qualitative changes (referred to as switching; Figures 29.7A and 29.7C) and quantitative changes (referred to as modulation; Figures 29.7B and 29.7D). Switching neurons dropped responses and/or they developed new responses to one or several odors. All switches observed with respect to the CS+ odor were recruitments; those to the CS− could be recruitment or loss of response. Modulating neurons increased and/or decreased their response rates to different

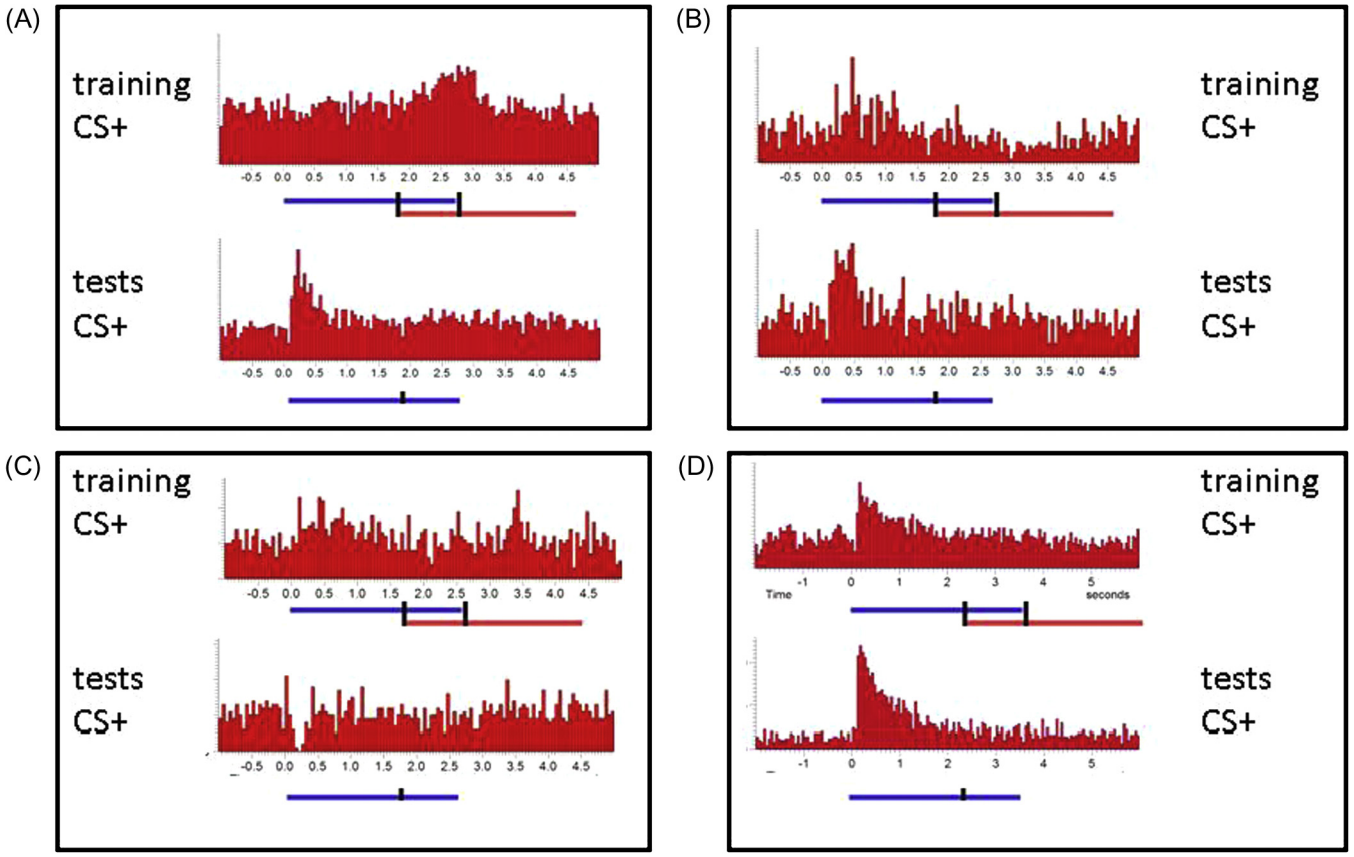

FIGURE 29.7 **Four types of associative plasticity in MB extrinsic neurons as they develop during olfactory reward conditioning.** The graphs give the sum of spikes during 10 repetitions of CS+/US pairing (training) or CS+ only (tests) for time bins of 50 msec. The top bar during conditioning and the bar during tests mark the CS+, and the lower bar marks the US during conditioning. The tick on the odor bar during tests marks the onset of the US during conditioning. (A) Before and during conditioning, the neuron responds only to the US; after conditioning, it responds to the CS+. This neuron is categorized as a switching EN. (B) The neuron responds before, during, and after conditioning to the CS+ and develops rather small quantitative changes of CS+ responding. This neuron is categorized as a modulating EN. (C) The neuron responds before and during conditioning with excitation to CS+; after conditioning, the CS+ induces an ON inhibition. This neuron is categorized as a switching EN. (D) The neuron responds before and during conditioning with phasic/tonic excitation to the CS+. After conditioning, the response to CS+ becomes more phasic. The time interval between conditioning and tests was 3 hr. This neuron is categorized as a modulating EN. Source: *After Strube-Bloss* et al.[60]

odors: CS+ always provoked increased responses, whereas CS− and control odors (the latter were used to test generalization phenomena) decreased or increased responses in approximately equal proportions. It was argued that the dichotomy of 'switching' and 'modulating' neurons may result from morphologically distinct ENs because switched and modulated neurons were rarely observed in the same recording. The delayed expression of associative plasticity in the switched and modulated ENs could reflect memory consolidation that depends on prolonged neural activities because consolidation in the MB can be blocked by cooling.

ENs appear to reflect both KC-related and own endogenous plasticity. The multiplicity of response changes during and after associative learning in several to many ENs could indicate a coding dimension at the MB output according to the meaning of the stimulus. Such meaning may be related to the prediction of the appetitive value, but it could also reflect different indicative categories of stimuli, such as a differentiation according to context and cue. Free-flying honeybees are known to learn the context in order to solve a discrimination task. They also learn contexts (light, colors, and temperature) quickly in the olfactory PER paradigm and use them for better discrimination.[62] They even master a trans-switching task in which the cue/context is reversed. ENs of the ventral α lobe close to the PE1 were found to reduce their responses to the cue (odor), whereas they increased their responses to the context (Figure 29.8). Therefore, these ENs do not simply code the appetitive value of a set of stimuli but,

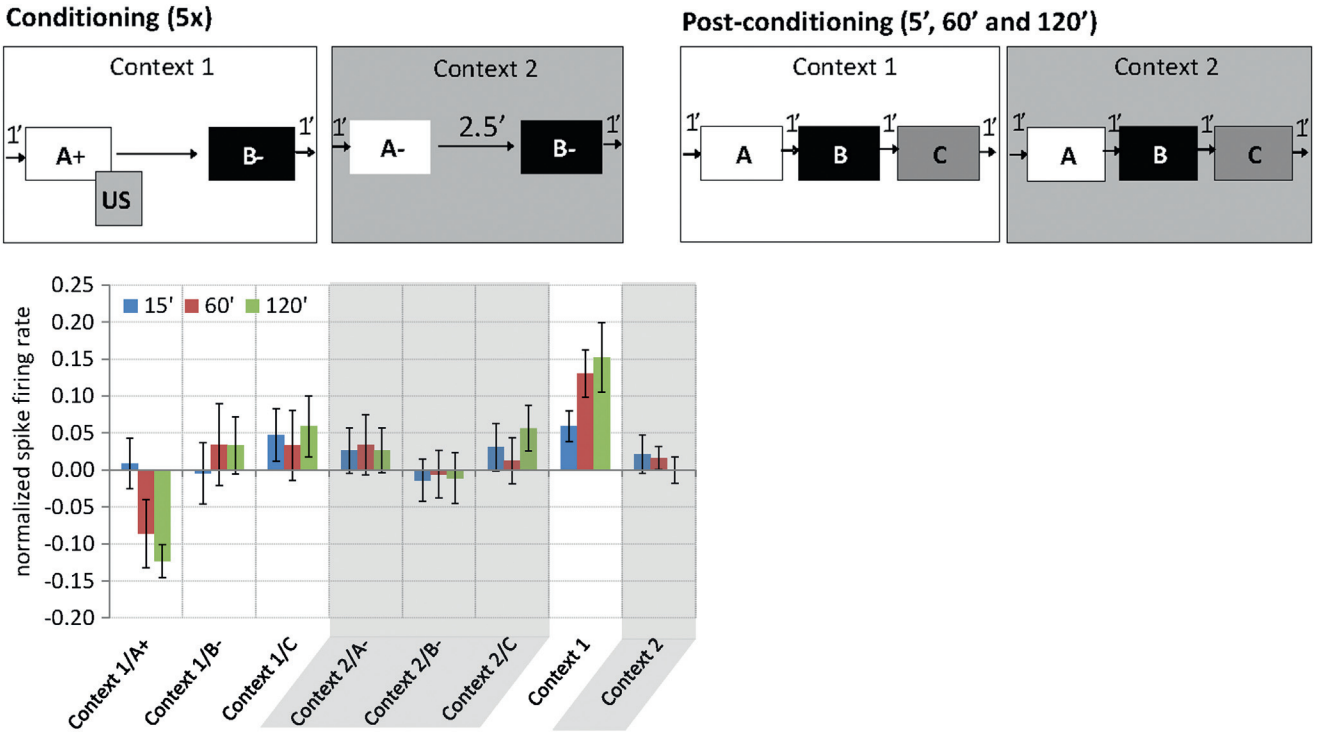

FIGURE 29.8 **Neural activity of ENs during context-dependent learning.** (Top) The procedure during conditioning and testing (post-conditioning). During conditioning, bees were presented with context 1 (bright light) and odor A was presented together with the sugar reward (US), whereas odor B was presented without any US. Then, context 1 was turned off and context 2 (dark) was presented, after which the two odors A and B were presented without any US. The five conditioning trials ended when context 2 was turned off. During testing (post-conditioning), each trial was presented once at 15, 60, and 120 min after conditioning. A trial consisted of context odor combinations or only context presentations. (Bottom) Normalized spike firing rate for different odor/context combinations as indicated. Spike firing rate toward odor A in context 1 was significantly reduced at 60 min ($p < 0.05$) and 120 min ($p < 0.01$) post-conditioning compared to the other two odors B and C. There was no difference in firing rate between odors A, B, and C in context 2. Firing rate toward context 1 increased at 15, 60, and 120 min ($p < 0.01$) after conditioning, whereas that toward context 2 remained unchanged. Plots show average normalized spike firing rate for each group, and error bars represent standard error of mean. Source: *After Hussaini and Menzel*.[62]

rather, differentiate according to a different dimension, cue and context.

Cue and context learning is represented differently in A3 neurons—those neurons in the GABA-ir PCT that serve inhibitory feedback locally in the α lobe and in a recurrent loop that projects back into the calyx (Figure 29.1). Ca^{2+} imaging experiments revealed increased neuronal responses to CS+ after training, decreased response to repeated odor stimulation attenuating specifically for the CS+, and decreased response that was strongest for the CS−. These neuronal changes were linked to the behavioral changes as seen in retention tests on the first[63] or the second[64] day. Multiunit extracellular recordings of both subgroups of A3 neurons (A3-v and A3-d) revealed qualitatively similar associative plasticity for the cue (odor) and the context (color) in a double discrimination task in which a particular color indicated a particular odor−reward association and another color an odor−nonreward association (Figure 29.9).[65] These A3 neurons develop their learning-related plasticity during acquisition or during the course of days—some on the first day, and others on the second or third day.

Two response profiles with respect to learning-related plasticity have been identified. Neurons either increased (as shown in Figure 29.9) or decreased (not shown) their rate responses to the reinforced cue and context, and they also expressed the inverse rate changes to the non-reinforced cue and context. The first group was tentatively related to A3-d neurons and the second group to A3-v neurons. These antagonistic rate changes were stronger when the animals were able to discriminate between the conditioned odors behaviorally, and they peaked at discrete time windows for different neurons over a recording period of up to 3 days. With their output within the lobes, A3-d neurons are expected to enhance inhibition locally on other MB lobe ENs (e.g., PE1) and thus may act as the source of learning-related CS+ response reduction documented for the PE1 neuron.[56] Reduced learning-related inhibition via A3-v neurons feeding back into the calyx might enhance synaptic strength in

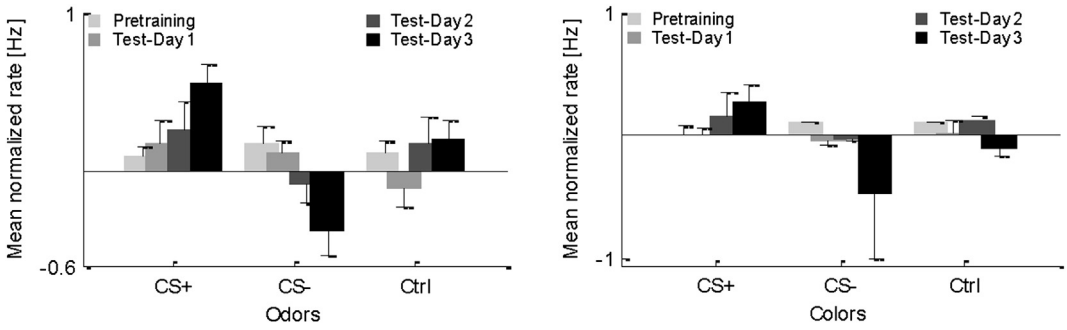

FIGURE 29.9 **Response changes of A3 neurons following context-cue training.** The graphs show responses separately for the cue (odors) and the context (colors) before training and during retention tests on three consecutive days. During retention tests, the specifically reinforced (CS+) odor (left) and color (right), the non-reinforced odor/color, and a control odor/color not presented during training were applied during extinction trials (tests without rewards). The graphs give the normalized spike rates during extinction tests separately for odors and colors of those neurons that developed their highest associative plasticity (the strongest rate difference between the conditioned stimuli) on the third day. Cues and contexts are represented in A3 neurons with qualitatively similar spike rate changes. Source: *After Filla and Menzel.*[65]

specific neurons in the MB main input microcircuits (e.g., that of the PN boutons[66]) through reduced inhibition. Decreased GABAergic input to KCs would consequently favor the induction of synaptic plasticity for reinforced stimuli. Evidence in favor of this hypothesis arises from the GABAergic anterior paired lateral (APL) neuron of *Drosophila*, which has striking morphological similarities with the A3-v cluster of the PCT.[67] The APL neuron suppresses and is suppressed by olfactory learning, suggesting that reduced inhibition promotes learning.[67] Reduced recurrent inhibition may also be related to an attention mechanism. In any case, the information stored in the recurrent pathway appears to modulate associative plasticity at a strategically important site, namely that part of the MB where precise coding of stimulus conditions (within and across sensory modalities) dominates.

THE LATERAL HORN

Olfactory and reward pathways also converge at the LH (Figure 29.1), suggesting that this structure may also form associative memory traces. The LH is part of the lateral protocerebrum, a rather unstructured neuropil with multiple subregions as indicated by different projection areas of PNs, ENs, and other protocerebral neurons.[28] The LH receives olfactory input directly from the AL via collaterals of the median and lateral tracts of PNs and indirectly via ENs of the MB (Figure 29.1). Some of the neurons extrinsic to the LH may contact descending neurons either directly or indirectly (e.g., in the case of the cockroach[68]; see Chapter 5), possibly allowing for rather direct sensorimotor loops. Such loops may underlie innate and fast odor-controlled responses such as pheromone-driven behavior,[69] but other views relate the lateral protocerebrum more closely to the MB and other high-order integrating centers in the insect brain.[70] The direct sensory premotor connections bypass and shortcut the olfactory pathways via the MB, and because MB ENs (e.g., PE1) also project directly to the LH, it has been suggested that the LH output is controlled by the experience-dependent signals from the MB. Thus, in addition to the possibly stereotypical sensorimotor connections, three sources of learning-related plasticity may control premotor output (and across brain connections)—plasticity in the AL, MB, and that intrinsic to the LH. Unfortunately, no data exist on LH function and plasticity in the honeybee. In *Drosophila*, the activity induced by fruit odors and pheromones in the LH is well segregated, suggesting a spatial organization with respect to odor classes,[71] but again learning-related plasticity needs to be demonstrated.

MEMORY TRACES IN THE REWARD PATHWAY

The neuron sufficient for the reinforcing function of sucrose in olfactory conditioning, the VUMmx1,[29] responds to a large range of stimuli before conditioning, but its response to sucrose stimulation at both the antennae and the proboscis is particularly strong and long-lasting (Figure 29.10A). The dendritic arbors suggested that it may be involved in the processing of olfactory information (Figure 29.1), and indeed it carries the reward signal in olfactory conditioning. Furthermore, during differential conditioning, the response to CS+ is enhanced, and that to CS− is reduced until no response is seen for the CS−. This finding indicates an excitatory memory for CS+ and

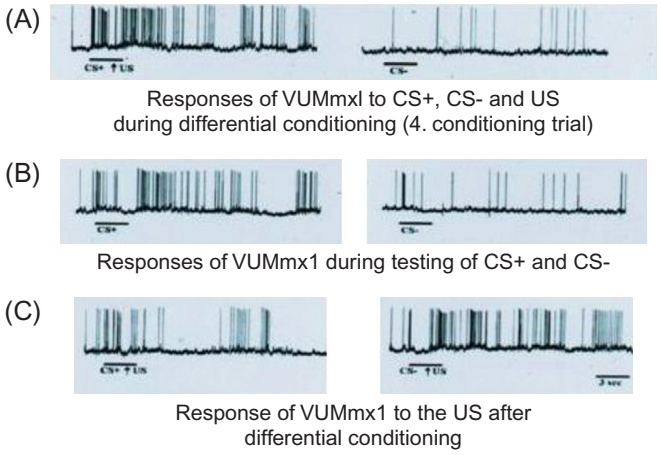

FIGURE 29.10 **Response characteristic of the VUMmx1 neuron to CS+, CS−, and US during and after differential conditioning.** (A) Initially, VUMmx1 responds to both odor stimuli (CS+ and CS−) and sucrose (US), but its response is particularly strong and prolonged to the US (sucrose). The responses during the fourth conditioning trial are stronger for the CS+, weaker for the CS−, and prolonged for the US. (B) In an extinction test, VUMmx1 responds strongly to CS+ and not to CS−. Note the similar response pattern to the CS+ and that to the US during conditioning. (C) If a US is presented after CS+, the sustained response is reduced, indicating that an expected US leads to inhibition of the sustained response, whereas an unexpected US as after CS− excites VUMmx1. Note the delayed burst of spikes occurring after both CS+/US and CS−/US stimulations.[72]

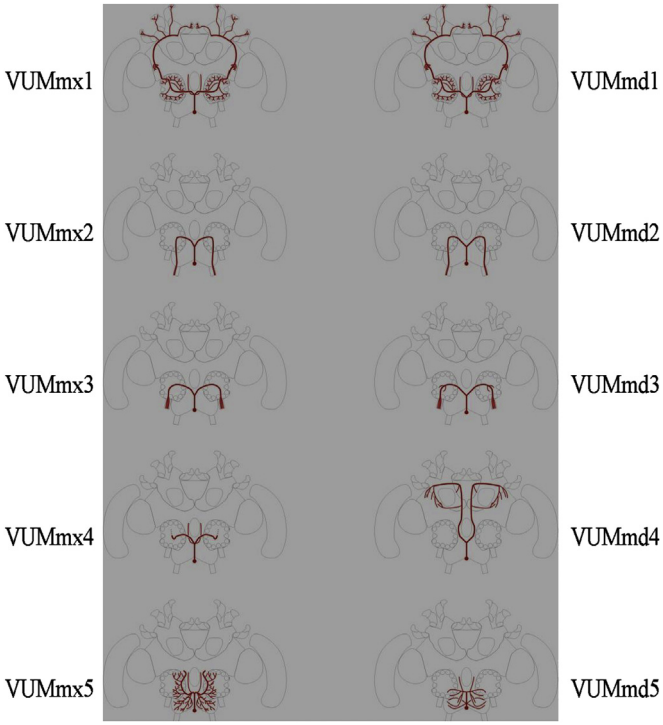

FIGURE 29.11 Five pairs of ventral unpaired median neurons immunoreactive to octopamin. Source: *After Schröter* et al.[73]

an inhibitory memory for CS− (Figure 29.10B). In addition, VUMmx1 develops a memory trace for the US because after conditioning and some consolidation period, an expected US induces inhibition in the sustained response. However, an unexpected US, such as after CS− application, excites VUMmx1 (Figure 29.10C). Interestingly, a delayed burst of spikes characteristic for the US long after its offset is seen in CS+-only stimulations and in CS+/US as well as CS−/US (unexpected reward) stimulations (Figures 29.10B and 29.10C).

VUMmx1 is one of 10 ventral unpaired OA-ir neurons of the subesophageal ganglion.[73] Five VUM neurons are localized in the maxillary and mandibulary neuromere and express pairwise corresponding dendritic structures (Figure 29.11). VUMmx1 and VUMmd1 match each other in dendritic arbors and response physiology; VUMmx2, VUMmd2, VUMmx3, and VUMmd3 send their dendrites along the antennal and mandibular nerves; VUMmx5 and VUMmd5 are likely to arbor in the antennal lobe and the ring neuropil around the α lobe (Figure 29.11 shows that they could not fully be reconstructed); and VUMmx5 and VUMmd5 innervate neuropils of the subesophageal ganglion. The responses to sucrose in most of these neurons make it likely that they carry information about the arousal and reward function of appetitive stimuli possibly under control of the level of satiation. Five additional corresponding VUM neurons have been reported for the labial neuromere. Because 6−8 OA-ir ventral cell bodies were seen in each of the three neuromeres of the subesophageal ganglion,[74] it is likely that there is a total of at least 18 VUM neurons in the bee brain. Assuming that all these VUM neurons are related to transmitting various components of appetitive stimuli and that as yet undetected VUM neurons reach all sensory (and possibly premotor) processing areas, it is likely that the positive value-based neural system is highly multifaceted. Additional aminergic neurons may participate in such an appetitively modulatory and reinforcing system, including particular dopamine neurons in the *Drosophila* brain. Each of these neurons or neuron pairs may store its own particular reward-related nonassociative and associative memory, and in combination with its respective target areas may constitute particular subsets of stimulus evaluation during learning, retrieval, and evaluation of stimulus compounds. It is thus not surprising that stimulation of octopamin receptors enhances feeding behavior, reward-seeking behavior, arousal, sensitivity to multiple sensory stimuli,[75] and social

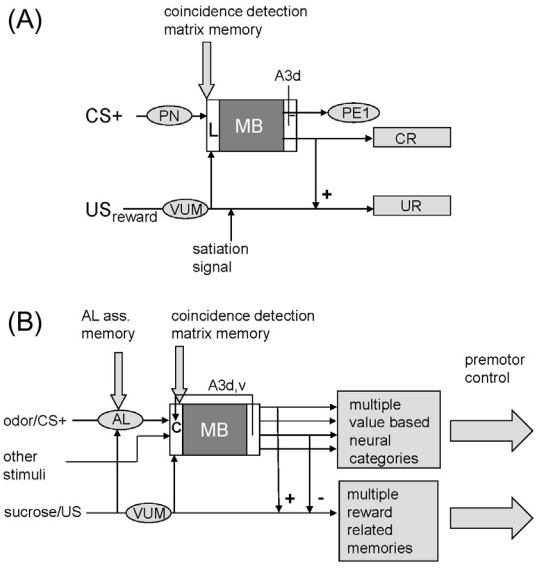

FIGURE 29.12 Two models of memory traces in the honeybee brain. (A) A radically simplified model. (B) A more adequate model.

interactions,[76,77] whereas blocking octopamin receptors reduces appetitive arousal, learning, and retrieval.[33,78]

THE DISTRIBUTED NATURE OF THE ENGRAM

Olfactory PER conditioning comprises a simple form of associative learning, but multiple processes at multiple sites are involved in forming the respective engram. The components observed so far are likely to comprise only a fraction of all associative changes comprising the full engram. It is also likely that these multiple sites are differently contributing to the sequential engrams during system's consolidation. Despite this complexity, it will be helpful for guiding future experiments to formulate a working hypothesis that attempts to integrate, at least partially, the current knowledge. Such a radically simplified model of the engram in the bee brain assumes a single, stable memory trace in the MB (Figure 29.12A). Spatial/temporal odor coding in the AL and the lip region of the MB calyx is thought to lead to an odor representation at the level of KCs by sparse and specific population activities. These network activities are associated with reward via convergence with the reward-encoding neurons (VUMmx1 and VUMmd1). Subpopulations of KCs differ with respect to their input from evaluating signals as indicated by specific gene activation patterns for octopamine and dopamine receptors[79]; thus, they are assumed to differ with respect to their coding scheme of different sensory modalities. It is therefore likely that the olfactory input needs to be distributed over the whole calyx, ensuring that the olfactory stimuli can be evaluated differently and combined with other stimuli in multiple combinations. Associatively enhanced activity in KCs driven by the CS+ will lead to stronger CS+ responses in ENs, as found in some ENs exciting the α lobe in its ventral and lateral aspect. The ENs expressing an inhibitory transmitter such as A3 neurons could inhibit other ENs such as PE1 within the lobes, reducing their CS+ responses. A subpopulation of ENs may transmit their enhanced CS+ excitation onto VUMmx1, causing the reward neuron to respond to CS+ and not to respond anymore to the US due to an intrinsic form of postexcitatory inhibition after it had responded to the CS+.

This radically simplified model could include various forms of nonassociative plasticity. For example, modulation of odor coding in the AL via VUMmx1 could lead to transient facilitation that could mimic enhanced odor responses following sucrose stimulation. Similarly, facilitation via VUMmx1 activity could counteract depression after stimulus repetition in KCs. Both modulatory phenomena could account at least partially for motivational and/or attentional effects induced by the appetitive stimulus in hungry bees. The associative effects observed in pre- and postsynaptic elements of the AL glomeruli would result in part from modulation via VUM neurons.

Such a radically simplified model of the memory trace offers a concept for explaining the recoding phenomenon in the MB from stimulus specificity to stimulus value based on the high divergence from PNs to KCs at the input and the extreme convergence of more than 100,000 KCs onto a few hundred ENs at the output. In addition, it assigns stable odor encoding to the AL, allowing the PNs to transmit an experience-independent odor code to the lateral protocerebrum and to the MB calyx. However, this model does not incorporate a whole range of findings both at the level of the AL and at the level of the MB. Furthermore, it is unconceivable that the enormously rich forms of learning in honeybees as seen under natural conditions and even partially in PER conditioning (context dependence and reward expectation) could be adequately conceptualized by such a simple model. A more realistic model of the distributed engram in the bee brain will have to incorporate the AL and the subtypes of KCs (and in further studies, the premotor and motor centers), and it needs to search for processing categories as they are read out from the MB. In this context, it will be helpful to consider the large range of connectivity patterns established by the ENs between the MB lobes and different brain parts.[28]

Evidence is strong for an independent memory trace in the AL. Although the stronger and more synchronized responses of PNs to the learned odor could result from feed-forward loops to the AL carrying information about the learned odor—for example, via the reward pathway (VUMmx1) or/and via inputs from the MB—additional assumptions are necessary to include the different forms of associative plasticity in glomeruli as seen in both Ca^{2+} imagining and multielectrode recordings. PNs are either up- or downregulated for the CS + depending on whether or not they responded to the CS + before learning. It is unlikely that such plasticity could result from VUMmx1 modulatory signals or from a single forward loop from the MB. The stable enhancement of odor mixture coding after learning requires plasticity of the odor coding network in the AL and cannot be provided by general modulatory signals. Additional arguments in favor of an independent olfactory memory trace in the AL come from US substitution experiments by local injection of octopamine into the AL and the finding that blocking of octopamin receptors in the AL interferes with olfactory learning. In addition, it was shown that the transition from short- to long-term memory can be facilitated by activating cAMP-dependent PKC in the AL. Taken together, both the MB calyx and the LH appear to receive odor signals from the AL that encode experience with the particular odors and counteract the concept that the AL codes odors in a stable and experience-independent way. However, what exactly is transmitted about experience is unclear. It may well be that it is limited to enhanced attentional effects rather than to indexing a specific odor.

The simplified model assigns the stimulus-specific memory trace to the associative matrix of divergent PNs onto KCs in the lip of the MB calyx and, more generally, to all neurons reaching the calyx and feeding the more than 100,000 KCs of the MB. The matrix memory could indeed store the rich content of the memory trace, including all relevant combinations of external (cues and contexts) and internal stimuli, because formally it could possess the necessary intrinsic properties—divergent and convergent connectivity, sparse population and temporal coding, and high thresholds in KCs—making them respond only to convergent input. Neuroanatomical evidence supports the assumption that all sensory inputs more or less processed converge in the MB calyx onto KCs. A tiny glimpse into such a storage device is given in Figure 29.4 for a specific subtype of KCs (clawed KCs of KC II) receiving input across the modality-specific regions (lip, collar, and basal ring) of the calyx. As mentioned previously, the different types of KCs combine different subsets of inputs, some of which keep the sensory modalities apart and others combine them. Modulating and evaluating pathways reach the calyx (VUMmx1 and VUMmd1) or the peduncle (neurons immunoreactive to dopamine and serotonin). Their pattern of convergence with the different KC types is unknown except for the fact that the two VUM neurons reach only the olfactory input (lip region of the calyx) and not other sensory inputs. It will be necessary to demonstrate how visual and other modalities are evaluated by reward and whether such an associative matrix-based model also applies to these sensory modalities. It is also not known how aversive stimuli are evaluated by the MB, except for the hint that dopamine neurons are likely to be involved,[80] which is also corroborated by findings in *Drosophila*.[81] Because the expression of aminergic receptor genes differs between groups of KCs, this indicates that subsets may be selectively involved in coding appetitive and aversive forms of learning.[79] These authors also provide evidence in favor of different subpopulations of KCs to store short- and long-term olfactory memory.

Important neural components of the calycal matrix memory also include the presynaptic sites—for example, the boutons of the PNs for olfactory memory. Because these microcircuits can be easily quantified histologically, their structural plasticity in the course of natural life history and olfactory learning is well documented, indicating a structural substrate of the lasting memory trace at the MB input site. Surprisingly, Ca^{2+} imaging during learning reveals rather small associative effects in the presynaptic boutons of PNs (Yamagata, personal communication). Hypothetically, protein synthesis-dependent restructuring of PN boutons as seen after olfactory conditioning[43] may be orchestrated by postsynaptic effects of KCs, and short-term associative effects may therefore not be seen. If this interpretation is correct, different patterns of change could correspond to short- and long-term memory traces storing the same content—a concept supported for the MB of *Drosophila*.[50]

The most deficient aspect of the radically simplified model of memory trace in the MB is the assumption that the readout of the matrix memory is limited to very few types of ENs establishing direct connection to premotor centers in the bee brain (e.g., the LH). An alternative view interprets the outputs as processing circuits that represent acquired and value-based categories of stimulus combinations and assumes multiple forms of reward-related memories stored in the VUM neurons (Figure 29.10B). Although the number of ENs is small compared to that of KCs, their structures and response properties are enormously rich. Both their connectivity patterns and their response changes during learning and memory formation indicate that ENs are involved differently in the readout of the MB. As noted previously (Figure 29.5), eight different groups

of ENs have been characterized on the basis of their somata loci and their arborizations patterns. The rich structural variability makes it likely that ENs of different groups serve different functions. Although these differences cannot be interpreted yet, the structural multiplicity suggests forms of combinatorial coding of neural processing categories that are defined by the respective input and output regions. What are these categories?

A common property of these categories could be that they represent acquired and value-based information. The following value-based categories (VBCs) come to mind:

1. Detection of novel versus already learned stimulus conditions
2. Distinction between appetitively and aversively learned stimulus conditions
3. Separation between cues and contexts
4. Separation between self-generated stimuli as experienced during active exploration and passively experienced stimuli as during classical conditioning
5. Storing stimulus traces for later learning, particularly under latent learning conditions as in navigation
6. Recognition and learning of symmetrical inputs to paired sense organs across sensory modality
7. Activation of specific memory traces for consolidation, such as during sleep or other forms of neural self-organization

These and other VBCs may define higher order neural processes brought about by the cooperation and combination of the lower level neural processes occurring in other parts of the brain, including (1) defining global context conditions (within the social context vs. acting individually; foraging for food vs. foraging for information during exploration), (2) working memory as a neural platform for the evaluation of expected outcomes, and (3) wakefulness versus sleep.

Although the assumption of defined VBCs is speculative, it may help in future work to relate some of the groups of ENs to these or other VBCs on the basis of their morphology. Consider several examples. First, A3-v projecting back from the lobes to the calyx could be involved in the distinction between novel and learned stimulus conditions and may be involved in facilitating the respective stimuli according to the response strength of the KCs. (2) ENs of groups A1–A4 are characterized by their branches in the ring neuropil of the same MB from which they receive input. Although we know nothing about the function of the ring neuropil, it could be that it houses long-term memory outside the active circuits of the MB, and neurons communicating between lobes and ring neuropil may be involved in memory consolidation and memory retrieval. Third, ENs connecting the two MBs in the two hemispheres of the brain (A6 and A7) may help to detect symmetrical stimuli across sensory modalities, transfer memory content from one MB to the other as proposed by Sandoz and Menzel,[82] and/or coordinate consolidation processes between the two MBs.

CONCLUSION

The engram of olfactory stimuli in the bee brain is characterized by its distributed nature with different prevailing processing categories at different sites. I conclude from the limited existing data that the trace in the AL relates predominantly to attention-generating properties, the matrix trace in the calyx to high-order combinatorics of all sensory inputs, the system of VUM neurons to appetitive internal states of the animal controlling nonassociative and associative traces, and the ENs of the MB to multiple processing categories that represent the acquired values and provide neural commands for goal-directed behavior and decision making. Although speculative, this framework offers a structure for experimental and modeling approaches and prevents us from believing that the properties of the memory trace can be captured by simply assuming flexible and experience-dependent sensory–interneuron–motor connections. Rather, we have to search for the coding/recoding, evaluating, and predicting processes involved in storing the contents of memory, the engram.

Gerber and co-workers[83] asked whether it is possible to localize a memory trace to a subset of cells in the brain. According to them, it needs to be shown that (1) neuronal plasticity occurs in the respective cells, (2) neuronal plasticity in these cells is sufficient for memory recall, (3) neuronal plasticity in these cells is necessary for memory formation, (4) memory content is lost if these cells do not function during retrieval tests, (5) and memory formation is abolished if these cells do not receive input during learning. This list of requirements, although difficult to meet experimentally (possibly only in *Drosophila* so far), is not complete and suffers from the focus on processes involved in neural plasticity rather than asking where and how the content of memory, the engram, is stored. The engram will not be found in a single type of neuron. It results from distributed network properties that add their respective contents when memory is formed, processed (consolidated), and retrieved. In other words, the engram is not a property of particular neurons but, rather, that of highly interacting networks of neurons. This form of interaction is different during memory formation, consolidation, and retrieval,

meaning that different engrams (for the same content) exist depending on what happens to them and for what they are used. In this way, the engram does not 'exist' but develops over time and in relation to actions of the brain as mirrored in incorporating new contents into existing ones, in consulting different contents during decision making and planning, and during execution of behavioral acts.

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