Cell. Mol. Life Sci. 63 (2006) 989–998
1420-682X/06/090989-10
DOI 10.1007/s00018-006-6024-8
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Cellular and Molecular Life Sciences

Dynamic memory networks: dissecting molecular mechanisms underlying associative memory in the temporal domain

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Online First 5 April 2006

Abstract. The molecular mechanisms underlying the induction and maintenance of memory are highly dynamic and comprise distinct phases covering a time window from seconds to even a lifetime. Neuronal networks, which contribute to these processes, have been extensively characterized on various levels of analysis, and imaging techniques allow monitoring of both gross brain activity as well as functional changes in defined brain areas during the time course of memory formation. New techniques developed in honeybees and fruit flies even allow for manipulation of neuronal networks and molecular cascades in a short temporal domain while a living animal under observation acquires new associative memories. These advantages make honeybees and flies ideal organisms to study transient molecular events underlying dynamic memory processing *in vivo*. In this review we will focus on the temporal features of molecular processes in learning and memory formation, summarize recent knowledge and present an outlook on future developments.

Keywords. *Drosophila*, honeybee, memory, antennal lobes, mushroom bodies, signaling cascades.

Introduction

In recent decades the cellular and molecular basis underlying learning and memory have been extensively studied in invertebrate species such as *Aplysia* [1], *Drosophila* [2–4] and the honeybee [5], but also in various vertebrate species [6–8]. Altogether, this search for the major molecular players provided compelling evidence that the mechanisms underlying learning and memory formation are highly conserved throughout the animal kingdom. A milestone was the finding that the signaling cascade mediated by cyclic AMP (cAMP) is a conserved player in memory formation in all species [9, 10], an idea based on early work in *Aplysia* and *Drosophila* [11–14]. However, in addition to the cAMP cascade, many other signaling cascades and molecules have been identified as equally important for learning and memory formation [articles in this multiauthor review]. Most of these molecular processes contribute quite specifically to distinct features of learning and memory formation and are tightly linked to the impact of the training procedure on memory formation. It is noteworthy that repeated and spaced training sessions can reliably induce long-term memory (LTM) or long-lasting neuronal changes [15–19] and that LTM, but not short-term memory (STM) or mid-term memory (MTM) requires protein and RNA synthesis [19–22]. However, other memory phases could be dissected on the basis of their underlying molecular requirement [23–25], directly raising the issue of how critical features of these defined memory phases can be identified. During the learning act, the molecular processes underlying induction of a distinct memory phase may only last a few seconds, whereas the phase itself may become evident only after several hours or days. Moreover, the induction and expression of distinct memory phases may be localized in different neuronal networks.

In this review we will focus on recent attempts to unravel this complex temporal and spatial network of cellular and molecular events implicated in various aspects of learning and memory formation. Particularly in *Drosophila* and in honeybees techniques are available that enable investigations with regard to the temporal and local func-

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tion of cellular and molecular networks *in vivo*. While *Drosophila* provides molecular techniques that allow manipulation of distinct molecular processes in defined neurons, the honeybee provides access to techniques enabling both monitoring and manipulation of signaling cascades during learning.

Olfactory conditioning in fruit flies and honeybees

Honeybees have been favorite subjects for behavioral studies since the turn of the century [26]; the breakthrough for neurobiological analysis, however, was the introduction of an associative conditioning paradigm, the olfactory conditioning of the proboscis extension response (PER) in the laboratory [27–29]. In this robust Pavlovian conditioning paradigm, an odor (conditioned stimulus, CS) is paired with a sucrose reward (unconditioned stimulus, US). Bees, although harnessed, form an association between the two stimuli, and a high percentage of animals will extend their proboscis after odor stimulation alone [5]. As in other species, the number of conditioning trials induces different memories which exhibit different properties. Memory induced by a single-trial conditioning (weak training) decays over time, is sensitive to amnestic treatments [30, 31], and is independent of translation and transcription. Repetition of the trials (3 trials within 4 min) induce a stable, long-lasting memory (>7 days) that is insensitive to amnestic treatments and requires translation and transcription [24, 32] (Fig. 3). Although several other learning paradigms use different sensory modalities, the vast majority of our knowledge is derived from investigations using the associative olfactory conditioning paradigm.

In *Drosophila*, the model system with the most sophisticated transgenic molecular and genetic tools, a naive olfactory cue can be associated with either a rewarding sugar stimulus or a punishing electric shock stimulus [33, 34]. Performance of the two memories – the rewarded sugar memory or the punished shock memory – can be tested for in an identical forced-choice situation. This unique experimental design enables investigation of the cellular and molecular substrates underlying each form of behavioral plasticity (reward learning or punishment learning) individually [4, 35, 36], and direct comparison of the mechanisms underlying each form of olfactory memory formation [37].

A similar neuronal circuitry underlies olfactory conditioning in bees and flies

The neuronal networks involved in odor processing are quite similar in *Drosophila* and honeybees (Fig. 1), including the antennal lobes (ALs), the mushroom bodies

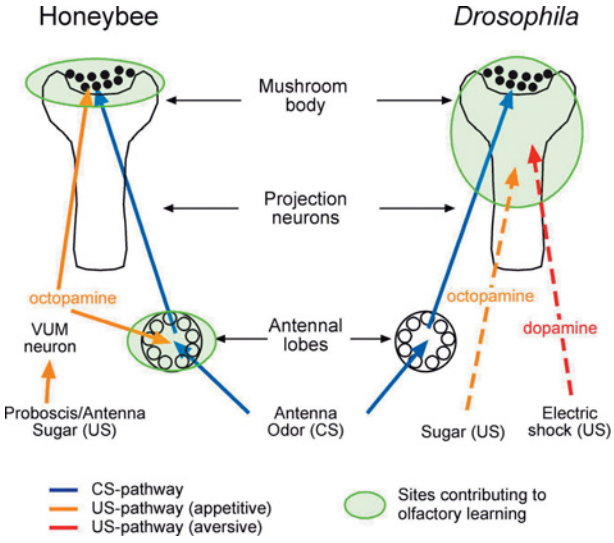

Figure 1. Neuronal circuitry involved in olfactory memory formation. Neuronal circuits mediating the odor (CS pathway) and sucrose information (US pathway) in honeybee and *Drosophila* olfactory learning. CS pathway: In both species the olfactory information received from the antennae is processed in the antennal lobes and relayed via projection neurons to the calyces of the mushroom bodies (MBs) and the lateral protocerebrum. US pathway: Sucrose reward information in associative appetitive learning requires octopaminergic transmission in both systems. In the honeybee the VUMmx1 neuron, involved in US processing aborises in the antennal lobes, the calyces of the MB and the lateral protocerebrum. Processing information of the aversive electric shock in *Drosophila* olfactory learning requires dopaminergic transmission. Sites demonstrated to contribute to olfactory learning and memory formation are highlighted in green.

(MBs) and the lateral protocerebrum. Olfactory information (CS) from the antennae project into the ALs, the equivalent of the olfactory bulb in vertebrates, and is then relayed via projection neurons to the calyces of the MBs and the lateral protocerebrum. In both species, the ALs are projection targets of the olfactory receptor neurons, and γ-aminobutyric acid (GABA)-ergic inhibitory interneurons might serve a first computation of the olfactory information to form an odor-specific topographic map [38, 39]. Projection neurons (PNs) relay this topographic odor map from the ALs to the lateral protocerebrum [40–43] and the MBs [44–46]. Moreover, in *Drosophila* this topographic pattern of PN activation undergoes changes during the time course of an aversive conditioning trial, resulting in recruitment of additional PNs into the representation of the shock-associated odor [47]. Yet the behavioral relevance of this short-lived neuronal plasticity within the PNs remains unknown.

In the case of the honeybee, processing of chemosensory information from the antennae and the proboscis, which acts as unconditioned stimulus (US) in the training, project to the suboesophageal ganglion and terminate near motor neurons involved in proboscis extension [48]. Ventral unpaired median (VUM) neurons receive chemosen-

sory input, and the VUMmx1 neuron – which converges with the CS processing pathway in the ALs, the MBs and the lateral protocerebrum – has been demonstrated to mediate US information in associative learning [49].The output from the MBs and the lateral protocerebrum, which drive the proboscis extension in associative learning, have not yet been identified. In the case of *Drosophila,* specialized gustatory receptors have been identified which send projections to distinct regions of the subesophageal ganglion [50–53]. From here, output tracts to higher brain centers are sparsely characterized, but for sugar reward learning in *Drosophila* the neurotransmitter octopamine is required [37], as is the case in honeybees [17, 54]. The preferential expression of octopamine receptors in *Drosophila* MBs [55] points to a potential function of octopaminergic VUM-like neurons in the processing of sucrose reward, as shown for honeybees [49]. Thus, appetitive olfactory conditioning partly relies on the same transmitters and utilizes similar cellular substrates in honeybees and fruit flies (Fig. 1).

Aversive conditioning is so far unique to flies and utilizes an electrifiable grid that covers the conditioning chamber [36]. By which receptors flies sense the electric shock applied during conditioning is completely unknown at the moment. However, for *Drosophila* to acquire an aversive olfactory memory, synaptic transmission from dopaminergic (DA) neurons must occur during the training period [37]. DA neurons send projections throughout the whole brain, including a tight innervation of the MBs at the heel region and the tips of the vertical lobes [37, 56]. Functional imaging of these DA neurons has revealed them to be strongly responsive to electric shock. Moreover, during the time course of an aversive conditioning trial these neurons acquire prolonged activity for the odor associated with shock. This finding and the fact that the MBs express several types of dopamine receptors [57, 58] is suggestive of a role for these DA neurons in processing and evaluating the aversive US in *Drosophila* olfactory conditioning [56].

MBs are essential for *Drosophila olfactory learning*

Anatomical evidence places the MBs at a central position of the CS processing pathway with prominent olfactory input into the calyx region and output neurons connecting the MBs to other brain areas [59–63]. Interestingly, the MB is necessary only for associative olfactory behavior [64, 65] and dispensable for spontaneous olfactory behavior [66]. This, and the fact that gene products of mutants with poor performance in associative olfactory learning and defects in the cAMP signaling pathway [14, 67, 68] are preferentially expressed in the MBs, pointed to a critical function of the MBs, in associative olfactory learning [55, 57, 69, 70]. Using *Drosophila's* sophisticated genetics directly tested this hypothesis.

The learning mutant *rutabaga* (*rut*) affects a type I adenylyl cyclase [14] and performs poorly in the olfactory associative paradigms [33]. In an attempt to identify the minimal circuitry that requires Rut function, Zars et al. [71] generated genetic mosaics expressing the wild-type *rut* complementary DNA (cDNA) only within the MBs and thereby restored proper cAMP signaling exclusively within that brain region. This genetic treatment (Fig. 2a) is sufficient to rescue performance of *rut* mutants to wild-type levels of either aversive or appetitive memory performance [37, 71]. Moreover, induction of *rut* function acutely within the adult MBs is also sufficient to rescue the memory defect, while restricting its expression to the developmental phase is not (for aversive memory). This finding rules out the possibility that *rut* mutants perform poorly due to a poorly developed brain (Fig. 2b) [72, 73]. The Rutabaga cyclase is activated via transmitter G-protein signaling and in addition by intracellular Ca^{2+}/calmodulin signaling. This dual activation leads directly to a model where the cyclase itself can integrate signals from the CS pathway and the rewarding or punishing US pathways in the MBs [3, 74, 75].

Controlling synaptic transmission in the temporal domain

Genetic evidence from *rut* rescue experiments suggest that olfactory memories are acquired in a cAMP-dependent fashion within the MB structure [37, 71, 73]. As proposed, cAMP signaling is involved in the initial step of memory formation, and therefore the localization is restricted to memory acquisition; so it remains unknown whether these memories are still located within the MBs at later times as they consolidate [33, 76] and perhaps become independent of cAMP signaling. Although unlikely, Rut might alternatively be involved in memory retrieval by yet unknown mechanisms. To address this question, the *Drosophila Shibire^{ts}* (*Shi^{ts}*) mutation was expressed in the MBs.

The *Shi^{ts}* mutation affects synaptic transmission in a temperature-dependent, dominant negative fashion, and ectopic expression of *Shi^{ts}* can disrupt synaptic transmission in the space of minutes and is nearly fully reversible in its effect [77]. This tool allowed taking MB signaling 'offline' during different phases of memory processing by shifting temperature from permissive (20–24 °C) to restrictive conditions (>30 °C). Blocking MB output during memory retrieval in the test situation prevented performance of aversive olfactory memories [78–80] as well as appetitive memories [37]. However, when trained under offline conditions, memory could still be observed as long as the MBs were brought back 'online' for memory retrieval during the test phase. These results suggest that retrieval of olfactory memories requires MB signaling ir-

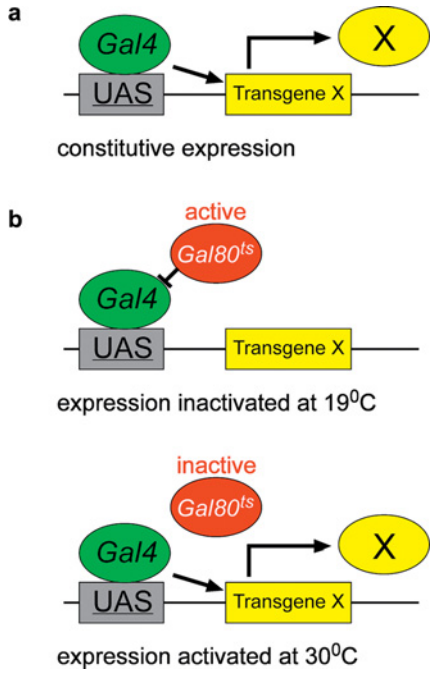

Figure 2. Transgenic tools for spatial and temporal analysis in *Drosophila*. *Drosophila* provides an unique system for ectopic transgene expression in defined cells. As with a toolbox genetic crosses can freely combine the single components of this system. (*a*) GAL4-UAS [108] is a two-component system utilizing the yeast transcription activator GAL4 to drive expression of any transgene cloned behind the upstream activating sequence (UAS). Each construct (the GAL4 and the UAS) is individually inserted into the genome of a *Drosophila* line. The genetic elements can interact in the genome of the offspring's after crossing the desired parental lines. Large collections of GAL4 driver – providing the cell-type specificity – and UAS-transgene lines are available from public stock centers. (*b*) The GAL80 ts construct [109] is a temperature-sensitive repressor of GAL4 action. At restrictive temperature (19 °C) GAL80 efficiently inhibits GAL4 from transcription, while at permissive temperature (30 °C) the inhibitor becomes inactive to allow GAL4-driven transcription. This construct is fully compatible with the existing collections of GAL4 and UAS lines.

respective of the US used during conditioning, and moreover that olfactory memories must be acquired upstream of the block induced by the *Shi*^{ts} mutation, i.e. upstream of MB output. To address the problems of memory consolidation, a series of experiments revealed that MB signaling remains necessary for aversive memory performance for up to 3 h [80]. Together with data on rescuing the *rutabaga* learning defect, these data strongly support the idea that in *Drosophila* aversive olfactory memories are formed and stored within the MBs for up to 3 h.

Neurotransmitter systems supporting distinct memory phases in *Drosophila*

Dopamine and octopamine differentially affect the formation, but not the retrieval or consolidation, of aversive and appetitive olfactory memories: using the *Shi*^{ts} transgene to bring dopaminergic neurons offline during the training phase prevented formation of the aversive olfactory memory nearly completely, whereas the reward memory remains unaffected. However, mutants devoid of the neurotransmitter octopamine [81] were unable to form associations after sugar reward but are unaffected in formation of aversive memory [37]. As both types of memory formation can be rescued by MB-specific expression of the *rut* cDNA, both types of olfactory memories must be formed in the MBs but depend on different neurotransmitters for US signaling during training.

Additionally, the cAMP pathway has been proposed to undergo prolonged activation via neuropeptide signaling [82]. In the *Drosophila* mutant *amnesiac* (*amn*) initial aversive memory formation is unaffected, but the memory exhibits an unusual steep decline within 30–60 min after training [68]. The gene affected in *amn* mutants encodes for neuropeptides, one with homology to pituitary adenylyl cyclase activating hormone (PACAP) [83], which is suggestive of a mechanistic link between the *amn* phenotype and cAMP signaling [82]. In *Drosophila*, the *amn* neuropeptides are expressed in two neurons, the dorsal paired medial (DPM) neurons that tightly ramify the lobe system of the Mbs [84]. Expression of wild-type *amn* cDNA within the DMP neurons was sufficient to rescue the *amn* mutant phenotype using aversive conditioning. Moreover, blocking synaptic transmission from the DPM cells by use of the *Shi*^{ts} transgene within a critical time window of 30–60 min after conditioning could phenocopy the *amn* mutant phenotype, resulting in fast memory decay [84, 85]. Functional imaging of these cells revealed that, unlike the dopaminergic neurons innervating the MBs, the DPM neurons are responsive to any odorant tested as well as to electric shock [86]. However, pairing of the odor with electric shock results in a delayed increase in synaptic release from DMP neurons only onto the vertical MB lobes, not the medial ones. This increase is specific to the shock-associated odor [86] and suggestive of a role of DPM signaling in aversive memory consolidation rather than acquisition. Thus, the circuitry involved in olfactory memory formation extends beyond MBs, as distinct transmitter systems seem to play unique roles at different time in establishing and maintaining distinct memory phases.

Local and temporal dissection of CS-US convergence in associative learning in honeybees

Knowledge of the neuronal circuits implicated in olfactory learning together with the accessibility of the honeybee brain allowed the application of amnestic treatments (cooling) immediately after learning to analyze the con-

tribution of distinct brain areas *in vivo* [30, 31]. While learning is affected by ALs cooling in an interval of <2 min after training, cooling of the MBs impairs learning in an interval <4 min after training. These early studies provided initial evidence of a contribution of MBs in associative learning. Moreover, these findings led to the hypothesis that ALs and MBs act partially independent and seem to contribute to different features of learning and memory formation. The idea that MBs and ALs are implicated in learning was elegantly supported by experiments using electrical stimulation of the VUMmx1, which innervates both neuropiles (Fig. 1) [49]. Depolarization of VUMmx1 shortly after CS presentation (forward pairing) can substitute for US function, while depolarization before CS presentation (backward pairing) is ineffective. The finding that octopamine is the putative transmitter of VUMmx1 [87] not only suggested an important role of octopamine in US processing but allowed local dissection of octopamine function in the honeybee brain. Pairing of an odor with subsequent local octopamine injections into either the ALs or the MBs can substitute for the US [17, 54]. Repeated pairing of CS presentation followed by octopamine injections into the ALs shows an acquisition phase, while pairing CS with injections into the MBs does not. Despite this difference, memory is formed in both cases as demonstrated by CS in the retention test 30 min after injections. Thus, ALs and MBs act partially independently with regard to associative learning and seem to contribute to different features of learning and memory formation.

Monitoring stimulus-induced dynamic activation of signaling cascades in defined brain areas of honeybees

The accessibility of the honeybee brain in combination with especially developed biochemical techniques provides the possibility to monitor *in vivo* induced changes in the activity of defined signaling cascades. Rapid freezing in liquid nitrogen after *in vivo* stimulation followed by a fast and specific biochemical assay [88–90] enables temporal resolution of less than 0.5 s. Limited by the accuracy of the dissection of distinct brain areas in liquid nitrogen, local resolution does not enable cellular analysis in the honeybee. However, it is possible to follow the dynamic activation of signaling cascades induced by learning in the time range of seconds to days in brain areas such as the ALs and distinct parts (input/output) of the MBs. This approach led to the identification of the second messenger systems that mainly mediate US and CS stimuli *in vivo*.

Sucrose stimulation which represents the US induces a very rapid (<1s) but transient increase in protein kinase A (PKA) activity in the ALs *in vivo* [88, 89], while odor stimulation (CS) or mechanical stimulation of the antennae has no effect on PKA activity in the ALs. This, together with other findings, provides strong evidence that US-induced activation of PKA in the ALs is mediated by octopamine released from the VUMmx1 neuron [88, 89]. Although MBs contribute to associative learning [17, 54] and their input areas, the calyces, are innervated by the VUMmx1 neuron, stimulation with sucrose (US) does not lead to PKA activation in the MB calyces *in vivo*. Thus, in contrast to ALs, octopamine receptors stimulated by VUMmx1 activation in the MB calyces are most likely coupled to Ca^{2+}-regulated pathways [91, 92]. In accordance with the broad innervation pattern of VUMmx1 neuron, determination of US-induced PKA activation in the ALs [88, 89] demonstrates a rather global activation within all glomeruli of the ALs. In contrast to this, odor stimulation (CS) induces a very specific glomerular activation pattern characteristic of each odor as demonstrated by Ca^{2+}-imaging techniques [93]. Although olfactory representation is sparser and sharper in the temporal domain [45], combinatorial activity patterns are also evident at the sites of olfactory input into the MBs.

Taken together, the spatial and temporal analysis of CS- and US-induced processes in the honeybee provides the basis for how these major inputs required for associative learning are processed in a neuronal network *in vivo*.

Induction of LTM: the critical temporal contribution of the cAMP/PKA cascade and glutamate in honeybees

As in other species, the cAMP/PKA cascade is required for formation of LTM. Blocking PKA activity during and shortly after associative conditioning specifically impairs LTM without affecting learning, STM or MTM [94]. The fact that LTM induction requires repeated training trials points to a close relation between stimulation parameters and induction of signaling cascades underlying LTM. This hypothesis has been directly addressed by measuring the temporal pattern of PKA activation induced by different training patterns *in vivo* [94]. According to observations with US stimulation alone, learning-induced changes in PKA activity are only detected in the ALs, but not in the calyces of the MBs. While a single CS/US forward pairing induces a transient elevation in PKA activity which lasts for less than 60 s, three CS/US forward pairings in succession prolong this PKA activation up to more than 3 min. Single or repeated backward pairings (US/CS) show the same temporal pattern of PKA activation as a single US stimulation [94]. The fact that honeybees can be trained while the brain is accessible by this system allows the unique possibility for manipulations such as locally and temporally defined PKA activation by photolytic stimulation of caged cAMP. Using this approach, caged cAMP was locally photolyzed in the ALs following single-trial conditioning, in order to artificially

prolong PKA activation. A local replay of prolonged PKA activation in the ALs combined with a single conditioning trial is sufficient to induce a long-lasting memory (Fig. 3). This finding provided direct evidence for a tight connection between conditioning parameters, temporal dynamics in PKA activation and its contribution to formation of LTM in intact animals.

The high temporal and spatial resolution of the uncaging technique allowed the characterization of the molecular mechanisms underlying the multiple-trial induced prolonged PKA activation. It turned out that the nitric oxide (NO) system, which is required for LTM formation in the honeybee [95], mediates prolongation of PKA activity via activation of soluble guanylate cyclase and cyclicGMP [94]. While inhibition of soluble guanylate cyclase blocks both the multiple-trial induced prolonged PKA activation and LTM formation; photorelease of caged cGMP in the ALs in combination with single-trial conditioning induces LTM, as uncaging of cAMP does. The initial suggestion that the NO-induced increase in PKA activity (≈25%) in the ALs is most likely mediated via cGMP by direct activation of PKAII is supported by the finding that cGMP can synergistically activate honeybee PKAII in presence of low cAMP concentrations [96]. Although the target of prolonged PKA activation is as yet unidentified, these measurements demonstrate that already in a short time window during conditioning a distinct temporal pattern of PKA activation is critical for LTM induction and thus for processes that become evident days later. However, since imitation of prolonged PKA activation in the ALs in conjunction with single-trial conditioning does not reach the level of conditioned proboscis extension reflex (PER) after multiple-trial conditioning, a contribution of other processes or brain areas is very likely.

Local release of glutamate in honeybee MBs revealed evidence of a function of glutamate in insect LTM formation (Fig. 3). Although glutamate plays a central role in neuronal plasticity in vertebrates [97], its function in the insect brain is poorly understood. The impairment of glutamate function by systemically applied pharmacological tools revealed evidence that glutamate is involved in insect learning; the results, however, are controversially discussed [98–100]. Using photolytic uncaging of glutamate *in vivo*, we recently demonstrated a spatial and temporally defined function of glutamate in insect learning [101]. Glutamate release immediately after a weak training protocol (single-trial training) in the MBs enhances formation of a late memory phase (2 days) and thus mimics the effect of a strong training protocol. Uncaging glutamate immediately before single-trial training in the MBs, or uncaging glutamate in the ALs does not affect memory performance. Thus, the action of glutamate is restricted to the MBs, where it contributes to induction of a late memory phase in a defined time window shortly after training. Although it is still unclear whether this late memory phase is mechanistically identical with LTM induced by strong training (three-trial conditioning), these findings provide the basis to test how glutamate action in the MBs and cAMP/PKA action in the ALs interact in processes of LTM formation.

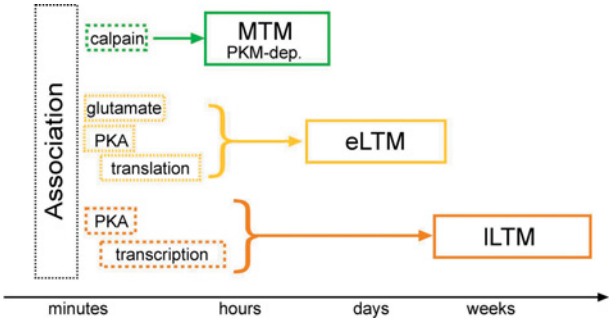

Figure 3. Parallel and serial memory phases in the honeybee. The scheme shows pharmacologically distinguishable memory phases in honeybees. The mid-term memory (MTM) requires protein kinase M (PKM) produced in a narrow time window after conditioning by cleaving protein kinase C (PKC) by calpain. MTM is formed independently and in parallel to long-term memory (LTM). LTM can be divided into an early translation-dependent phase (eLTM) and a late phase (lLTM) that depends on transcription. Induction of LTM (eLTM and lLTM) requires a defined PKA activation pattern in the antennal lobes mediated by nitric oxide (NO). Glutamatergic transmission in the MBs facilitates the formation of LTM. The finding that lLTM can be induced in absence of eLTM points to the existence of two parallel and independent PKA-induced processes during conditioning.

Different parallel molecular processes contribute to memory formation

Disruption of molecular processes by pharmacological and genetic tools in various species provided clear evidence that memory formation is a continuous and dynamic process (Fig. 3). Although training paradigms may differ, in all cases at least three memory phases can be identified: STM in the range of minutes, MTM in the range of hours, and stable LTM which last for days and weeks [102]. LTM in honeybees can be divided into an early phase (eLTM, 1–2 days), which requires protein synthesis, and a transcription-dependent late phase (lLTM, ≥3 days) [24, 32, 103]. Both LTM phases seemed to be triggered by a single cAMP/PKA process, since blocking PKA activity during the training period leads to loss of both eLTM and lLTM [94, 104]. Although this hypothesis is in accordance with other systems [105, 106], detailed analysis considering the impact of satiation level revealed evidence of a more complex function of the cAMP/PKA cascade in LTM induction [107]. While three-trial conditioning of hungry animals leads to induction of all memory phases, additional feeding 4 h before

three-trial conditioning is sufficient to impair acquisition and induction of all memory phases. Different basal PKA levels in honeybee brains at different satiation levels pointed to a contribution of the cAMP cascade. Rescuing low basal PKA activity in animals fed 4 h before conditioning specifically rescues transcription-dependent lLTM, while acquisition, MTM and eLTM are still impaired. This supports the existence of two parallel pathways triggered by the cAMP/PKA cascade during conditioning: one triggers molecular events leading to translation-dependent eLTM; the other process triggers a cascade responsible for transcription-dependent lLTM (Fig. 3).

Determination of learning-induced modulation of Ca^{2+}-phospholipid-dependent protein kinase C (PKC) in the honeybee uncovered another parallel acting system in memory formation [24]. Although PKC-mediated processes play a role in synaptic plasticity in vertebrates, their function is not well investigated [108]. Both US and the CS induce transient PKC activation in the ALs of the honeybee [24]. Since inhibition of PKC activity during the conditioning phase does not affect acquisition or memory formation, PKC activity during conditioning seems not to be involved in induction of processes required for memory formation. However, measurements of PKC activity hours and days after training revealed a new function of PKC in memory maintenance. Three-trial conditioning triggers an increase in PKC activity in the ALs starting 1 h after training and lasting up to 3 days. This elevation of PKC activity can be dissected into two phases. In the early phase (1–16 h), a constitutively active PKC, the PKM, contributes to the elevated activity. PKM is formed by cleavage of activated PKC by Ca^{2+}-dependent protease calpain (Fig. 3). Calpain inhibition during conditioning prevents PKM formation and impairs memory in a time window between 1 and 16 h. PKC blockers do not affect acquisition, early memory at 30 min or memory after 1 day. This and the fact that late training-induced elevation of PKC is also unaffected by calpain inhibition demonstrates that PKM formation in the ALs is an independent parallel process required for maintenance of this MTM phase. The function of the mechanistically independent increase in PKC activity observed in the late phase (1–3 days), which depends on RNA and protein synthesis, is yet unclear. It is probably one of several mechanisms acting in parallel that occur in different brain areas but are required for the formation of the late phase of LTM.

Future prospects: increasing resolution in the temporal domain

The *Drosophila* GAL4/UAS system [109] has facilitated access to the neuronal networks underlying learning and memory by providing a high degree of spatial resolution (see Fig. 2a). Unfortunately, this system discounts for temporal control of transgene expression, a shortcoming which can partly been compensated for by adding the temperature-sensitive $GAL80^{ts}$ construct as a third component (see Fig. 2b, [110]). However, the temporal resolution of this tripartite system is not suited to probe the fast molecular mechanisms underlying memory formation in the temporal domain. Conditional transgenes, such as as the Shi^{ts} transgene discussed above might represent an alternative as they are faster, in the range of minutes, but the caveats are obvious as Shi^{ts} is limited in its functional specificity to synaptic transmission, and useful conditional alleles are rare and hard to identify.

To date, the best temporal resolution can be achieved by focal uncaging of signaling molecules from inactive compounds at the expense of cellular specificity, as these compounds cannot be expressed by genetic means. A combination of these two approaches provides both a high degree of temporal resolution and cellular specificity in *Drosophila:* Lima and Miesenbock [111] have recently used the GAL4/USA system to express ligand-gated ion channels in defined neurons that usually lack them. These neurons can then produce action potentials upon illumination as the flash of light liberates the specific agonist from a caged precursor that has to be applied systemically. Directing expression of this channel to the giant fiber system is sufficient to elicit specific behaviors upon illumination; the fly can, so to speak, be remotely controlled. Another elegant system has been provided by Nagel and colleagues [112]. The authors used *Caenorhabditis elegans* to transgenically express a channel rhodopsin, a directly light-gated channel from green algae, in mechanosensory neurons. Upon light illumination the worms exhibit withdrawal behaviors usually elicited by mechanical stimulation. These new techniques based on light-activated proteins allow quick and reversible activation of neuronal activity within the space of seconds. In the near future these powerful tools will help to dissect the neuronal circuitry involved in associative memory formation.

Acknowledgements. This work was supported by the Deutsche Forschungsgemeinschaft.

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