Learning in honeybees: from molecules to behaviour**

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Summary

Studies in a variety of organisms as diverse as molluscs, insects, birds and mammals have shown that memories can exist in a variety of temporal domains ranging from short-term memories in the range of minutes to long-term memories lasting a lifetime. While transient covalent modifications of proteins underlie short-term memory, the formation of long-term memory requires gene expression and protein synthesis. Different intracellular signalling cascades have been implicated in distinct aspects of learning and memory formation. Little is known however, about how learning in intact animals is related to the modulation of these signalling cascades and how this contributes to distinct neuronal and behavioural changes in vivo. Associative learning in the honeybee provides the opportunity to study processes of memory formation by analysing its progression through different phases, across levels of behaviour, neural circuits, and cellular signalling pathways. The findings reveal evidence that various cellular signalling pathways in the neuronal circuit of distinct brain areas play a role in different processes during learning and memory formation.

Key words: antennal lobes, associative learning, memory, protein kinases, second messenger cascades

Introduction

Memory formation is a dynamic process consisting of different phases which are induced and maintained by different mechanisms. The neural substrate of both short-term memory (STM) and long-term memory (LTM) is believed to reside in the synaptic connections between neurones. It has been convincingly demonstrated that second messenger cascades play a central role in the modulation of neuronal activity and the connectivity between neurones.

In various systems, including molluscs, insects, and mammals, the second messenger cyclic AMP (cAMP) and the cAMP-dependent protein kinase (PKA) play critical roles in processes of neuronal plasticity and learning. Early evidence of the role of the cAMP cascade in learning derived from the genetic analysis of the Drosophila mutants. The mutants dunce and rutabaga with defects in associative learning also have defects in enzymes regulating the cAMP level (for reviews see Davis, 1996; Dubnau and Tully, 1998). The cAMP/PKA cascade's role in learning and memory formation has been confirmed by investigations addressing the role of PKA and cAMP-responsive transcription (Davis, 1993, 1996; Dubnau and Tully, 1998; Yin and Tully, 1996), providing convincing evidence that the cAMP/PKA pathway plays a key role in the formation of LTM.

At about the same time, the cAMP/PKA pathway's role in formation of long-lasting neuronal changes has been demonstrated for the marine snail Aplysia. Here, the induction of synaptic long-term facilitation (LTF), a cellular analogue of a defensive withdrawal reflex, requires the cAMP-mediated processes (Byrne and Kandel, 1996; Carew, 1996). The fact that the cAMP is also

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**Presented at the 95th Annual Meeting of the Deutsche Zoologische Gesellschaft in Halle/S., May 20–24, 2002

0944-2006/02/105/04-313 $ 15.00/0

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essential for inducing of long-lasting neuronal changes in the hippocampus in mammals (Frey et al., 1993; Huang et al., 1995; Abel et al., 1997) suggests a conserved function of cAMP-mediated mechanisms with regard to formation of long-term memory throughout the animal kingdom. Recently it has been demonstrated that this is indeed the case. In all systems tested so far, the cAMP-dependent transcription mediated via the cAMP response element binding protein (CREB) plays an essential role in the transformation of STM into LTM (Yin and Tully, 1996; Silva et al., 1998). This and the fact that transition of STM into LTM strongly depends on the number and pattern of the training sessions calls for a connection between training pattern and activation of CREB-mediated gene expression (Müller and Carew, 1998; Müller, 2000). While the requirement of the cAMP/PKA pathway in LTM formation seems to be conserved throughout the animal kingdom, the contribution of other signalling pathways to learning and memory is not so well understood. One of these signalling cascades is the protein kinase C (PKC) pathway, which has been implicated in later stages of memory in vertebrate studies. Although many studies demonstrated changes in PKC activation and relocation consecutive to associative learning (Olds et al., 1990; Van der Zee et al., 1992) no conclusion can be drawn as to whether these mechanisms are also responsible for PKC modulation in associative learning in vivo. In summary however, most of the investigations indicate PKC’s contribution to memory expression at later stages rather than its function during the training phase (Roberson et al., 1996). While these studies demonstrate that distinct molecular components are required for distinct aspects of neuronal plasticity and learning, it is mostly unknown how these components are modulated by learning and memory formation and how this, in turn, contributes to the behavioural level. Given the fact that memory formation is a continuous multiphasic process, we must propose that the underlying molecular mechanisms are also highly dynamic. Moreover, formation of memory, and thus the underlying molecular mechanisms, critically depends on features like the number and the temporal succession of conditioning trials. Thus, to unravel the connection between the behavioural and the molecular levels, it is essential to understand the dynamic events at the molecular level that are induced as a consequence of learning and memory formation.

**Associative learning in honeybees: The olfactory conditioning paradigm**

The complex behaviour of honeybees, including orientation, foraging and social communication, has attracted interest since the turn of the century (von Frisch, 1967). Learning odours, colours, and flowers shapes is essential for bees and reveals many characteristics of associative learning described in mammals (Menzel, 1990, 1999; Menzel and Müller, 1996). A breakthrough for the neurobiological analysis was the introduction of associative olfactory conditioning of the proboscis extension response (PER) under laboratory conditions. A hungry bee extends its proboscis reflectively when the antenna or the proboscis receive an appetitive stimulus. In the olfactory learning paradigm, an odour, the conditioned stimulus (CS), is paired with a subsequent sucrose reward as the unconditioned stimulus (US) (Fig. 1A). The animals form an association between the two, so that a high ratio of bees will extend their proboscis after odour stimulation alone (Kuwabara, 1957; Bitterman et al., 1983). This olfactory learning by harnessed bees has all the features of an associative learning and is comparable with that observed in vertebrates (Menzel, 1990; Hammer and Menzel, 1995; Menzel and Müller, 1996). The number of conditioning trials applied to the honeybee induces different memories, which exhibit different properties (Fig. 1B). The memory induced by a single conditioning trial decays over several days and is sensitive to amnestic treatments. This memory is independent of translation and transcription. In contrast, multiple conditioning trials induce a stable, long-lasting memory (>7 days) that requires translation and transcription (Gründbaum and Müller, 1998; Menzel, 1999). This resembles characteristics that are also observed in other species. In *Drosophila*, *Aplysia*, and mice, the induction of transcription-dependent long-lasting neuronal changes and LTM also requires repeated training sessions or repeated spaced stimulations (Tully et al., 1994; Kogan et al., 1997; Sutton et al., 2002).

**Neural circuits implicated in olfactory learning**

The brain areas and the circuits implicated in the processing of odours are well characterised for the honeybee (Fig. 2). The olfactory information from the chemosensory receptors on the antennae project into the glomeruli of the antennal lobes. The glomeruli are sites of dense synaptic connections between sensory neurones, local interneurons and projection neurones. A few thousand local interneurons connect the 160 glomeruli within the antennal lobes. The olfactory information is then relayed via projection neurones to the calyces of the mushroom bodies and the lateral protocerebrum. Projection neurones with the olfactory information terminate on Kenyon cells in the lip region of the calyces. The outputs of the Kenyon cells are connected with other brain areas via the α and β lobes of the mushroom bodies. Thus the antennal lobes, the lateral protocerebral lobe
and the mushroom bodies are sites involved in the processing of olfactory information. These are exactly the neuropils that are innervated by the VUMmx1 (ventral unpaired median neuron maxillare 1) that plays a central role in US processing (Hammer, 1993). The VUMmx1 can substitute for the US function, as demonstrated by depolarisation of VUMmx1 shortly after a CS presentation. Octopamine is the putative transmitter of VUMmx1 (Kreissl et al., 1994), suggesting an important role of octopamine in US processing. This function could be demonstrated, since pairing of an odour with subsequent local octopamine injections into either the antennal lobes or the mushroom bodies can substitute for the US (Hammer and Menzel, 1995, 1998). There is however, a characteristic difference between octopamine injection into the antennal lobes and the mushroom bodies. While multiple pairings of CS and octopamine injections into the antennal lobes lead to a normal acquisition, injections into the mushroom bodies do not. In both cases however, the animals show a conditioned response in the retention test after twenty minutes. A difference in the contribution of the antennal lobes and the mushroom bodies to learning has also been observed after local cooling experiments (Erber et al., 1980; Menzel et al., 1974). These

![Diagram](image-url)

Fig. 1. Olfactory conditioning of the proboscis extension reflex in honeybees. (A) Scheme of the associative conditioning of the proboscis extension reflex (PER). Honeybees were harnessed in small metal tubes and fed to satiation. The next day the animals were conditioned by pairing an odour stimulus (CS) with a subsequent sucrose reward (US) to the antenna and proboscis. Memory retention is tested by stimulation with the odour alone. (B) One conditioning trial leads to formation of a memory that decays over days. Multiple conditioning trials (3 trials with an inter-trial interval of 2 min) lead to the formation of a long-term memory that is stable over days.

![Diagram](image-url)

Fig. 2. Neuronal circuits involved in associative olfactory learning in the honeybee. The neuronal circuits processing the odour stimulus (conditioned stimulus, CS) and the sucrose reward (unconditioned stimulus, US) in PER conditioning are well described. CS pathway: Olfactory information from the antenna is processed in the antennal lobes which are connected to the calyces (ca) of the mushroom bodies and to the lateral protocerebrum. The mushroom bodies, with their intrinsic Kenyon cells, process input from different sensory modalities. US pathway: The information from chemosensory receptors for sucrose on the antenna and the proboscis is relayed to the VUMmx1 neuron. The VUMmx1 neuron aborises in the antennal lobes, the calyces of the MB and the lateral protocerebrum and can substitute for the US. Thus the antennal lobes and the mushroom bodies are convergence sites of the CS and US pathways. The output pathways that connect the lobes of the mushroom bodies to the motor circuits of PER are unknown.
findings suggest that the antennal lobes and the mushroom bodies act partially independently with regard to associative learning and seem to contribute to different features of learning and memory formation.

Memory formation and the underlying signalling pathways

At the behavioural level learning includes processes covering time periods from seconds for the CS/US association up to weeks for LTM. Consequently, the underlying molecular mechanisms are supposed to act in corresponding time domains. Memory formation is an ongoing dynamic process that consists of different molecular mechanisms acting serially and in parallel. But since the induction of a distinct molecular process may require only a few seconds (e.g., during conditioning), its consequences may be visible only after several hours or days (e.g., LTM). Thus, identification and characterisation of the role of a distinct molecular process with respect to memory formation demands analysis over a long period of time. Due to the knowledge of the convergence sites of CS and US processing in the honeybee, the analysis of the molecular mechanisms underlying learning and memory focuses on the antennal lobes and the mushroom bodies.

Signalling cascades involved in the CS and US processing

Octopamine, the putative transmitter of the VUMmx1 neuron that plays a central role in US processing, causes a transient activation of the PKA in the antennal lobes (Hildebrandt and Müller, 1995a) (Fig. 3). A similar PKA activation is induced by sucrose (US) stimulation in vivo, while odour or mechanical stimulation of the antennae do not affect PKA activity in the antennal lobes (Hildebrandt and Müller, 1995b). The PKA is mainly localised in interneurons within the glomeruli (Müller, 1997). Due to the extensive aborizations of the VUMmx1 neuron, the US-induced octopamine release most likely modulates the cAMP/PKA cascade in local interneurons within all glomeruli. Although serotonin and dopamine are also detected in the antennal lobes, they do not affect PKA activity in the antennal lobe circuitry (Hildebrandt and Müller, 1995a).

In contrast to the antennal lobes, in vivo stimulation with sucrose (US) does not lead to changes in PKA activity in the mushroom body calyces that are innervated by the VUMmx1 neuron. However, octopamine and other biogenic amines are capable of activating PKA in cultured Kenyon cells (Müller, 1997). Since different subtypes of octopamine receptors are coupled to different second messenger pathways, the receptors stimulated by octopamine released from VUMmx1 are rather coupled to Ca\(^{2+}\) regulated pathways than to the cAMP pathway.

The processing of odours in the antennal lobes has been studied extensively using Ca\(^{2+}\) imaging techniques (Joeges et al., 1997; Galizia and Menzel, 2000) (Fig. 3). In contrast to the general modulation of the entire antennal lobe by the VUMmx1 neuron, each odour induces a characteristic glomerular activity pattern. Thus, in the antennal lobes, the first convergence site of US and CS pathways, the US induces a rather general activation of the cAMP/PKA cascade in the glomeruli, while the CS leads to a specific activation of a few glomeruli.

Fig. 3. Signalling pathways implicated in CS and US processing in the antennal lobes. Sucrose (US) stimulation of the antenna leads to an increase in cAMP levels in the antennal lobes mediated via VUMmx1 and octopamine. This in turn causes a transient activation of the cAMP-dependent protein kinase A (PKA). Odour (CS) stimulation of the antenna leads to changes in Ca\(^{2+}\) levels and transiently activates the Ca\(^{2+}\)/phospholipid-dependent protein kinase C (PKC). Imaging techniques show that this Ca\(^{2+}\) increase is restricted to a distinct subset of glomeruli that are characteristic for each odour. It is still unknown which target proteins are phosphorylated by PKA and PKC with respect to CS and US processing.
The induction of LTM: The critical role of the cAMP/PKA cascade

In the honeybee, as in other species, blocking PKA activity during associative conditioning results in a loss of LTM without affecting learning or memories in the hours range (Müller, 2000). Several studies suggested that stimulation parameters required for LTM induction lead to a characteristic PKA activation pattern, that, in turn, is required for LTM induction. To test this hypothesis, the temporal dynamics of PKA activation induced by different conditioning patterns were determined using rapid freezing after in vivo stimulation followed by a fast and specific PKA assay (Hildebrandt and Müller, 1995a, b; Müller and Carew, 1998).

Learning-induced changes in PKA activity could be detected in the antennal lobes, but not in the calyces of the mushroom bodies (Fig. 4A). A single CS/US forward pairing, which does not induce LTM, leads to a transient increase in PKA activity in the antennal lobes that returns to basal levels 60 s after the conditioning trial. This transient elevation of PKA activity is drastically prolonged after the third CS/US forward pairing, which induces LTM. A single US/CS backward pairing or multiple US/CS backward pairings induce the same temporal PKA activation as a single US stimulation. Thus the temporal dynamics of PKA activation in the antennal lobes depend on the temporal pairing of CS and US and on the number of conditioning trials applied (Müller, 2000). The NO system, which is required for LTM formation in the honeybee (Müller, 1996), mediates prolonged PKA activation in the antennal lobes during multiple-trial conditioning. Local imitation of the prolonged PKA activation in the antennal lobes by photorelease of cAMP in combination with a single conditioning trial is sufficient to induce LTM (Fig. 4B). This strongly supports the idea that a training procedure that induces LTM leads to a temporal pattern of PKA activation necessary for LTM formation. It also demonstrates that a distinct PKA activation in the antennal lobes only a few minutes after conditioning is critical for LTM induction and thus for processes that are realised days later.

The most likely target for the prolonged PKA activation induced by multiple-trial conditioning is the cAMP/Ca\(^{2+}\)-regulated transcription, via cAMP response element binding protein (CREB) (Yin and Tully, 1996; Abel et al., 1998). The CREB protein family consists of isoforms that act either as activators or repressors of gene expression. This, together with the phosphorylation of CREB by various protein kinases regulates CREB-mediated gene expression. In all systems tested so far, CREB plays a central role in the transition from short- to long-lasting neuronal changes and in the induction of LTM (Abel et al., 1998; Silva et al., 1998).

LTM in Drosophila, which is induced by repeated spaced training sessions, and LTF in Aplysia which requires repeated 5-HT stimulation, are selectively blocked by repressor isoforms of CREB. In contrast, an excess of activator isoforms of CREB is sufficient to induce LTM in Drosophila or LTF in Aplysia under

Fig. 4. LTM formation depends on prolonged PKA activation during conditioning. (A) Inhibition of PKA activity during conditioning leads to a loss of LTM which is only induced after multiple conditioning trials. The temporal dynamics of PKA activation in the antennal lobes differ, depending on the training pattern. A single conditioning trial that does not induce LTM leads to PKA activation that returns to the baseline (dashed line) within 60s. Three conditioning trials that induce LTM lead to a significantly prolonged PKA activation in the antennal lobes (arrows). (B) The prolonged PKA activation measured after three-trial conditioning was imitated by locally uncaging cAMP in the antennal lobes. A single trial conditioning, which is not sufficient to induce LTM, combined with the artificially prolonged PKA activation in the antennal lobes, is able to induce LTM in vivo (adapted from Müller, 2000).
conditions which usually induce only short-term changes (Silva et al., 1998). However, the complete sequence of events that connect the training procedure with CREB-mediated gene expression required for LTM formation has not yet been identified yet. Since regulation of CREB activity is the result of a complex interaction of different second messenger systems, it is very likely that different signalling cascades contribute to LTM formation. The distinct pattern of PKA activation in the antennal lobes caused by an LTM inducing training pattern is therefore a very early but critical event in the signalling cascade leading to LTM formation in vivo.

Processes maintaining mid-term memory: The function of protein kinase C

Calcium is the most common signal transduction molecule that controls many aspects of cellular function. Among numerous Ca^{2+}-regulated processes, protein phosphorylation mediated via Ca^{2+}-phospholipid-dependent protein kinase C (PKC) plays a major role in synaptic plasticity. Although changes in PKC activity following learning suggest that PKC plays a role in memory formation (Olds et al., 1990; Van der Zee et al., 1992), the findings are controversial and not as conclusive as those confirming the conserved function of the cAMP/PKA system in LTM induction. In contrast to the US specific activation of the cAMP cascade in the antennal lobes, PKC is activated by both the US and the CS (Grünbaum and Müller, 1998). The temporal pattern of PKC activation is independent of the sequence of CS and US stimulation and the number of conditioning trials. Inhibition of these transient PKC modulations during the conditioning phase affects neither learning nor the formation of olfactory memory. Thus, PKC activity during conditioning is not required for learning and memory formation but may be involved in chemosensory information in the antennal lobes.

However, the detailed analysis of the learning induced changes in PKC activity covering a long period of time identifies PKC’s contribution to memory formation (Fig. 5). Hours after olfactory conditioning, a specific learning induced modulation of PKC activity is obvious in the antennal lobes. In contrast to a single conditioning trial, multiple trial conditioning that induces LTM leads to an increase in PKC activity beginning 1 hour after conditioning and lasting up to 3 days (Grünbaum and Müller, 1998). Two independent and different mechanisms contribute to this training-induced increase in PKC activity in the antennal lobes. In the early phase ranging from 1 to 16 hours, a constitutively active PKC, the PKM, is formed by cleaving the activated PKC by the Ca^{2+}-dependent protease calpain. The inhibition of calpain during conditioning prevents PKM formation and leads to a memory impairment ranging from 1 hour to 16 hours. Acquisition and memory after 1 day is not affected. Thus, formation of PKM in the antennal lobes seems to play a central role in the maintenance of this mid-term memory phase.

Fig. 5. Mid-term memory requires PKM formed during conditioning. Following induction of LTM by three-trial conditioning PKC activity in the antennal lobes is increased from 1h up to 3days. Increase in PKC activity in the range of hours is due to the production of constitutive PKM by proteolytic cleaving of PKC. Inhibition of the protease during the time period of conditioning (shaded area) leads to the loss of PKM and thus a reduction of PKC activity to the level of untrained animals (dashed line). Inhibition of PKM formation also leads to a reduction in memory retention in the same time period. Learning, STM in the minutes range and LTM in the days range are not affected by the loss of PKM (adapted from Grünbaum and Müller, 1998).
The increase in PKC activity observed in the late phase ranging from 1–3 days depends on RNA and protein synthesis and is unaffected by blocking the early phase. The specific contribution of this late phase to memory formation 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 long-term memory.

Towards an understanding of the dynamic processes of memory formation

Studies in humans provided early evidence that formation of memory is continuous and dynamic (Ebbinghaus, 1885). This initial study, as well as numerous subsequent studies in a variety of species, demonstrated that memory formation depends on training parameters (e.g., number and temporal relation between learning trials) and is sensitive to interference during its consolidation (e.g., by amnestic treatments). A major breakthrough in identifying memory phases was the introduction of pharmacological and genetic tools (summarised in DeZazzo and Tully, 1995). These treatments disrupt distinct molecular processes and thus demonstrate their contribution to memory formation. The comparison between different species reveals characteristic similarities with respect to different memory phases. At least three phases can be separated: A first phase is in the range of minutes, a second in the range of hours and a third in the range of days. In all systems the short- and mid-term phases do not require protein synthesis, while the long-lasting phase does (Fig. 6).

Our investigations of the learning induced modulations of second messenger cascades in the honeybee provide additional information with regard to the temporal dynamics of these signalling cascades. So it becomes clear that the induction and expression phases of a distinct molecular process may be separated by many hours. In the two examples presented (prolonged PKA activation and PKC to PKM conversion), induction occurs in parallel and independently during the few minutes of conditioning (Fig. 6). While the PKM is required for maintenance of a mid-term memory in the range of 1–16 hours (Grünbaum and Müller, 1998), the consequences of the prolonged PKA activation during conditioning are evident only after the first day (Müller, 2000). In the latter case the sequence of the signalling events and the molecules that contribute to the expression are unknown. It is feasible that different sequential and parallel mechanisms occurring in distinct brain areas are induced by the prolonged PKA activation during conditioning. This hypothesis is supported by more recent findings demonstrating that signalling cascade activity is required for LTM formation at distinct time windows after the conditioning phase. Such a complex network of signalling cascades would provide a perfect target to interact with the entire process of memory formation by inputs such as stress, circadian rhythm and competing memories.

Acknowledgements

I would like to thank R. Menzel for helpful suggestions on this manuscript and M. Wurm for help with the manuscript. This research was supported by the Deutsche Forschungsgemeinschaft.
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