The Biology of Memory

Symposium Bernried, Germany
October 15th–19th, 1989

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F. K. Schattauer Verlag · Stuttgart – New York
Chemical Codes of Learning and Memory in Honey Bees

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Summary

Behavioral pharmacological experiments with the honey bee, *Apis mellifera*, were performed to study whether the biogenic amines dopamine (DA), noradrenaline (NA), octopamine (OA) and serotonin (5-HT) are involved in the formation and retrieval of olfactory memory. The bees are conditioned to an odorant stimulus by one or a few trials, and the test substances are injected in miniature amounts directly into the brain. The effect of neuropharmacca on memory formation was tested by injections prior to conditioning (storage test), and on memory retrieval by injections after conditioning (retrieval test).

NA and OA appear to be general facilitatory modulators for both storage and retrieval but also for other aspects of chemo-sensory related behaviors, such as feeding, and sensitization by the unconditioned stimulus. 5-HT antagonises the action of OA and NA; memory storage is retarded and retrieval is reduced. DA, however, selectively effects the retrieval processes when injected into the median part of the brain (close to or in the central complex).

Cyclic AMP appears to be an important second messenger in the process of olfactory memory formation and retrieval. OA, NA and DA stimulate the adenylate cyclase in brain homogenate, but 5-HT shows either a slight stimulatory effect (crude homogenate) or an inhibitory effect (membrane fraction of homogenate).

We conclude that the biogenic amines DA, NA, OA and 5-HT function as important regulators of memory storage and retrieval in the bee brain. The action is, at least in part, mediated by cAMP. Here it would appear that modulatory change in cAMP concentration is the key parameter.

Zusammenfassung

Verhaltenspharmakologische Modellstudien an der Honigbiene *Apis mellifera* werden zur Untersuchung der Frage durchgeführt, welche Rolle die biogenen
Amine Noradrenalin (NA), Octopamin (OA), Dopamin (DA) und Serotonin (5-HT) bei der Bildung und dem Abrufen des Gedächtnisses spielen. Dazu werden die Tiere mit einem oder wenigen Lernakten auf einen Duft konditioniert und die Testsstoffen in geringen Mengen direkt in das Gehirn injiziert. Die neuropharmakologische Wirkung auf die Bildung der Gedächtnisspur wird durch eine Injektion der Testsstoff vor der Konditionierung geprüft, wobei der Lernvorgang zu einem Zeitpunkt stattfindet, an dem die pharmakologische Wirkung optimal ist (»Einspeichertest«). Die Wirkung auf das Abrufen aus dem Gedächtnis wird durch Injektion nach dem Lernvorgang geprüft (»Abrufetest«). Der Zeitverlauf in beiden Testverfahren wird durch mehrmäßige Tests verfolgt.

NA und OA wirken als generell anregende Modulatoren, sowohl für die Speicherung und Abrufung des Gedächtnisses als auch für stereotype Verhaltensweisen wie die Saugaktivität und die Sensitisierung durch den Zuckerstimulus. Serotonin wirkt in die andere Richtung, indem es die Einspeicherung und das Abrufen hemmt. DA zeigt einen selektiven Blockierungseffekt auf das Abrufen aus dem Gedächtnis, wenn es in den medianen Bereich des Gehirns (»Zentralkomplex«) injiziert wird.

Cyclisches AMP stellt sich als eine wichtige sekundäre Botensubstanz heraus. OA, NA und DA stimulieren die Adenylatcyclase in Gehirnhomogenaten, dagegen hat 5-HT entweder einen sehr geringen stimulatorischen Effekt (ungereinigtes Gesamthomogenat des Gehirns) oder einen inhibitorischen Effekt (Membranfraktion des Gehirnhomogenats).

Aus den Ergebnissen schließen wir, daß die im Bienengehirn auftretenden biogenen Amine DA, NA, OA und 5-HT eine wichtige modulatorische Rolle bei der Gedächtnisbildung und beim Abrufen aus dem Gedächtnis haben. Zumindest ein Teil dieser Wirkung wird über cAMP vermittelt. Dabei spielt wahrscheinlich die Auf- und Abregelung des cAMP-Gehalts und nicht so sehr die tatsächliche Konzentration des cAMP die entscheidende Rolle.

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**Key Words:** Conditioning – insects – honey bee – biogenic animals – cAMP.
Introduction

Neural plasticity, learning and memory are general features of nervous systems. Since nervous systems of even widely different animal species are evolutionary related, it is very likely that basic features of the cellular and molecular processes underlying neural plasticity, learning and memory, are common in many or all nervous systems. This reasonable assumption has led to the research strategy of posing the relevant questions to model systems in the most suitable preparations. Invertebrate species are particularly valuable, because they provide the experimenter with certain fortunate conditions. Molluscs, having big and relatively few neurons, are exceptionally suitable for electrophysiological and cellular techniques (see Byrne, Sweat, Bailey, this volume). *Drosophila* is a renowned subject for classical and molecular genetic studies, and memory mutants have been successfully used to elucidate a great number of important aspects of the substrate of memory (see Dudai, Tully, this volume). The honey bee *Apis mellifera* is particularly cooperative in behavioral studies; bees can be trained quickly and effectively, they establish a long lasting memory within very few learning trials and they can be collected from a colony with several thousands of genetically closely related animals in unlimited number at any time of the year [v. Frisch (28), Menzel (15)].

We have taken advantage of these conditions in bees and applied the concepts of behavioral neuropharmacology to study the following questions:
1. Which transmitters, neuromodulators and second messengers may be involved in learning and memory of the honey bee?
2. Do neuromodulators act specifically on behavioral categories of learning and memory?
3. Where in the brain and at what time during memory formation and memory retrieval do they exert their action?

The rationale behind behavioral neuropharmacology is that the cellular and molecular events underlying learning and memory involve cascades of reactions which are mediated by key substances, namely transmitters, modulators and second messengers. Manipulating these substances by pharmacological means should alter the behavioral measures of learning and memory. The alterations should be indicative of the potential role of the respective substance in the process of the cellular events. There are many pitfalls in this approach which have been discussed by many authors over the years [see for example Martínez (14), Dunn (5)]. However, it is our hope that the reduced complexity and highly stereotyped behavior of the honey bee offer suitable conditions for the strategy of behavioral neuropharmacology, which would help elucidate general features of the cellular machinery underlying learning and memory.
The behavioral paradigm

Olfactory learning in honey bees is a particularly fast associative learning process, and has thus been selected as the experimental paradigm for these studies. A single appetitive learning trial with a floral odor changes the behavior of a freely flying bee so drastically that it will later choose this odor more than 90% from among other odors [v. FRISCH (28), KOLTERMANN (10)]. The same fast learning and stable memory is also found when bees are harnessed in small tubes and prepared for appetitive conditioning (see Fig. 1). Bees extend their tongue (the proboscis) reflexively when the antenna is touched with sucrose solution. This proboscis extension reflex (PER) can be conditioned to an odorant (conditioned stimulus, CS) if the odor stimulus is presented shortly before the reflex is released and the animal is allowed to suck a small amount of the sucrose solution (unconditioned stimulus, US) [KUWABARA (11), MENZEL et al. (18)]. PER-conditioning is an

Fig. 1. Honey bees are fixed to a small metal tube and conditioned to an odorant stimulus. The bees extend reflexively the proboscis when the antennae are touched with a sucrose solution. The bees will associate the odor with the sucrose reward if the odor is presented shortly before the release of the reflex and the delivery of the sucrose solution to the proboscis. This fast associative learning process is also observed when the frontal head capsule is opened and the brain exposed. Glass capillaries with neuropharmacological test substances can be positioned into specific brain regions and nl-amounts of the substances locally applied.
associative learning process [Bitterman et al. (3)], in which only forward conditioning (CS precedes the US) is effective [see Menzel (17) for review]. As with freely flying bees, a single learning trial is sufficient to change the response to the CS from 5–10% before conditioning to 50–70% after conditioning. The memory established by a single learning trial lasts longer than 24 hours, and a sequence of several learning trials results in life-long memory. The memory trace following a single learning trial proceeds through temporal phases which are characterized by their susceptibility to experimental procedures such as cooling, narcosis, electric stimulation [Menzel et al. (18), Erber et al. (8), Menzel and Sugawa (20), Menzel (16)], and by the contribution of sensitization by the US. A model of the formation of a stable memory trace in bees which integrates the various results assumes a sequence of three memory phases and two different kinds of consolidation processes linking the three phases [see Menzel (17) for review].

The neuropharmacological test procedure

The experiments reported here will concentrate on the question as to which biogenic amines and second messengers are involved in the establishment and retrieval of an associative olfactory memory trace. For this the experimental animals were fixed in tubes, as shown in Fig. 1, the day before experimentation. The drugs were injected either in relative large quantities (30–200 nl) into the midbrain via a nerve tract which runs from the median ocellus to the upper part of the brain, the protocerebrum, or locally into different regions of the protocerebrum in small quantities (2–8 nl).

The brain or cerebral ganglion of the bee has a volume of approximately 1 μl and consists of 850,000 densely packed neurons (Fig. 2). Chemosensory information conveyed by the antennal nerve (SN) is processed by the antennal lobe (AL), a primary sensory structure, and in the motor centres for the mouthparts, the suboesophageal ganglion (SOG) and in two regions of the protocerebrum: the mushroom bodies (MB) and the lateral protocerebrum (LP) (see Fig. 2a and b). The mushroom bodies are essential structures for the consolidation of an olfactory memory [Erber et al. (8)] and will, therefore, be of importance for our pharmacological studies. The relay neurons from the antennal lobe (mAGT) project to the input region of the MB, the calyces, where the information is processed and integrated with inputs from many other sensory inputs in the 170,000 kenyon cells (K) in each mushroom body. The output neurons leave the mushroom body at the α- and β-lobe (α, β). The reflexive or conditioned motor response (proboscis extension) is controlled by motoneurons in the SOG, and these receive inputs from descending interneurons (dN) which connect to the output neurons of
the mushroom body. The US is conveyed via sucrose receptors at the tip of the proboscis, their afferent projections to the SOG and ascending neurons to the mushroom bodies. The antennal lobe, the median calyces and a-lobe of the mushroom bodies are easily localised and penetrated by capillaries from the front side of the brain, and thus its neuropil can be pharmacologically manipulated by local injections.

Fig. 2. a: Schematic representation of the brain (cerebral ganglion) of the bee (see text). Structures treated with local injections of neuropharmacae are indicated by stippled areas: C = median calyx of the mushroom bodies, a-L = a-lobe of the mushroom bodies, A-L = antennal lobe. Additional abbreviations: M = medulla, second visual neuropil, L = lobula, third visual neuropil, LP = lateral protocerebrum. b: Schematic wiring diagram of the neural connections relevant for the FER and its olfactory conditioning (see text). Abbreviations: SN = sensory nerve of the antenna, MN = motoneurons to the mouthparts, AL = antennal lobe, dN = descending interneurons from the brain to the SOG, SOG = suboesophageal ganglion, mAGT = median antennal glomerularis tract, second order projections from the antennal lobe to the MB, MB = mushroom body, K = Kenyon cells, intrinsic neurons of the mushroom body, α and β = α- and β-lobes of the MB, ex = extrinsic neurons of the MB.
Two experimental protocols were applied to study the question whether drugs interfere selectively with either formation or retrieval of memory (Fig. 3). In the "retrieval test" the animals were trained by a conditioning trial (CT) 10 min before injection (inj.), and tested three times by presenting the CS alone afterwards (T1, T2, T3). In the "storage test" the animals were first injected with a drug and conditioned at a time when the drug action is known to be optimal (between 5 and 20 min after injection depending on the drug). The three tests (T1, T2, T3) followed 20, 35 and 50 min later. The probability of proboscis extension to the CS alone (tests T1–T3) is taken as a measure of memory. Control groups were treated exactly the same but only the solvent (ringer solution) was injected. In the case of the two examples shown in Fig. 3, the difference between the experimental and the control group is presented (ΔR = % response of test group, % response of control group). In both cases the conditioned response is enhanced by the drug, only transiently in the retrieval test with synephrine (syn 10⁻⁴ M), but permanently in the storage test with noradrenaline (NA 10⁻⁸ M) (see below). The experimental and control groups consisted of 20 resp. 40 animals.

**Biogenic amines are eminently involved in modulating learning and memory in bees**

The bee brain contains DA, NA, OA and 5-HT in considerable amounts [Mercer et al. (22)]. The distribution of these amines strongly suggests a modulatory function rather than a function as fast acting local transmitters, although this cannot be entirely ruled out. Immunocytochemical studies showed that DA [Schäfer and Rehder (25)] and 5-HT [Rehder et al. (24)] appear predominantly in neurons with large arborisations sometimes covering neural volumes of nearly half the brain [Bicker et al. (2)]. Global injections of these amines into the brain modulate memory formation and retrieval in a dose dependent fashion, and/or sensory and motor components of the trained behavior [Erber and Kaulen (7), Michelsen (23), MacMillan and Mercer (13), Mercer and Menzel (21), Menzel et al. (19), Bicker and Menzel (1)]. A general finding of these early studies was that DA interfered with the retrieval of memory, NA and 5-HT inhibit both formation and retrieval of memory, and OA had no effect on the memory measures. With respect to sensory and motor components, 5-HT and OA appear to be functionally antagonistic modulators, 5-HT reducing the behavioral measures, OA enhancing them. DA blocked the sensory processing if the antennal lobes were treated selectively.

Additional evidence for the role of these amines comes from two series of recent experiments with L-Dopa and reserpine. Fig. 4 shows experiments in which
Fig. 3. The two experimental protocols of this study. a: Retrieval test: two groups of animals (controls and test, each 30–40 in number) were conditioned by one trial (CT) and 10–20 min later injected with 5 nl into each mushroom body. The experimental group received synephrine (10^-5 M), an OA-receptor agonist, and the control group ringer solution. Each bee was tested three times (T1, T2, T3) by presenting the conditioned odor (carnation) without the unconditioned stimulus (US, sucrose solution) at 3, 10 and 40 min after injection (abscissa). The ordinate gives the difference ΔR(%) between the experimental group and the control group. In this example, the tested drug enhances the percentage of conditioned responses (+ ΔR%) indicating a facilitatory effect of synephrine on the retrieval of the memory. b: Storage test: The two groups of animals are injected before the learning trial, the experiment animals with the test substance (here NA 10^-8 M, 2 nl in each α-lobe of the mushroom bodies), the control group with ringer solution. The conditioning trial (CT) is given at a time interval when the drug exerts its optimal action (here 10 min after injection). The three test trials (T1, T2, T3) are performed in 10 min intervals beginning at 30 min after injection. Abscissa and ordinate as above. The positive ΔR(%) indicates a facilitatory effect of the NA-treatment. Statistics: differences above 12% are significantly different on the p ≤ 0.05 level.
reserpine was injected either into the brain or into the body, and the animals were subsequently trained (either 30 min or 24 h after injection). In both cases learning of an olfactory stimulus is significantly reduced. Recent HPLC-measurements and immunocytochemical studies showed that body injection of reserpine causes a substantial (>70%) depletion of all biogenic amines from the brain tissue of insects [Brookhart et al. (3a, 4)].

Feeding L-Dopa to animals leads to increased levels of DA and NA. We have examined whether the conditioning behavior is effected by preceding L-Dopa treatment. Fig. 5 shows that the learning behavior improves in a dose-dependent fashion, in animals with relatively low learning rates (see control group). The reason for the low learning rate in this particular experiment is unknown, but it is obvious that in DA and/or NA enrichment after feeding of L-Dopa has a strong and highly significant enhancing effect.
feeding L-Dopa in sucrose
16h before

conditioning trials

Fig. 5. The effect of feeding L-Dopa to a group of animals, which showed a relatively low rate of conditioning. The plot shows the acquisition function of the control group ("sucrose") and three experimental groups (L-Dopa $10^{-2}$, $10^{-3}$, $10^{-4}$ M). The animals were fed with a sucrose solution 16 h before the conditioning experiment. In the case of the experimental group the sucrose solution contained the amount of L-Dopa as indicated ($10^{-2}$, $10^{-3}$, $10^{-4}$ M). The number of animals in each group is given with the right panel. (This experiment was carried out by the students on a course in Neurobiology at the Freie Universität Berlin 1989 under the supervision of R. Menzel.)

**Dopamine (DA)**

DA was found to be present in relatively large quantities in the bee brain [20 pM mm$^{-3}$, Mercer et al. (22)]. DA-like immunoreactive neurons are present in most parts of the brain and SOG [Schäfer and Rehder (25)]. 330 DA-immunoreactive somata were counted in each brain hemisphere, and the respective neurons invade particularly intensively the mushroom bodies and the unpaired central complex. The antennal lobes are penetrated by two widely branching DA-immunoreactive neurons. A common feature of all DA-immunoreactive fibres is that each fibre invades large volumes of neuropil with fine processes having a varicose appearance. This suggests that DA is more important in mediating distant and/or modulatory rather than local neural interactions.

Injection of DA in physiological concentrations ($10^{-5}$–$10^{-6}$ M) after conditioning leads to a transient reduction of the conditioned responses [Menzel et al. (19), Michelsen (23)]. Fig. 6 shows three different experiments, in which DA was either
Fig. 6. Three different series of experiments in which the effect of DA injections was tested on the retrieval of stored olfactory information. Experiment 1 (open squares): 5–10 nl of 10−6 M DA were injected close to or into the central complex (experimental group, n = 13; control group, n = 11) 20 min after 4 conditioning trials. Experiment 2 (close circles): about 10 nl 10−5 M DA were injected through the median ocellar nerve close to the central complex (experimental group; n = 29, control group, n = 26) 20 min after 5 conditioning trials. Experiment 3 (close squares): about 100 nl 10−6 M DA were injected through the median ocellar tract close to the central complex (experimental group, n = 68; control group, n = 64) 20 min after 2 conditioning trials [redrawn from Michelsen (23)]. All animals were tested several times at intervals indicated at the abscissa. The ordinate gives ΔR% as defined in the text. (Experiments 1 and 2 were performed by students on the Neurobiology course at the Freie Universität Berlin 1989 under the supervision of R. Menzel.)

injected in large quantities (30–200 nl) via the median ocellar tract (experiments marked with OcN) or in small quantities (5–10 nl) locally injected close to or into the central complex (experiment CB). In all three cases a significant reduction of the conditioned responses was found 20–30 min after injection, which fully recovered after 60 min. Most interestingly, no effect was found in storage tests in which the single or multiple learning trials were performed 20 min after injection, the time of optimal action of DA. Furthermore, no change in the strength or pattern of the reflex behavior or sucrose intake was observed [Menzel et al. (19), Michelsen (23)]. It is tempting to conclude from these results that DA releasing neurons particularly in the median protocerebrum (central complex) are involved in the motor expression of learned behavior, rather than the actual establishment of a memory trace or the sensory or motor components of the reflex.
Octopamine (OA)

OA is a prominent modulatory transmitter in the central nervous system and in the periphery of arthropods [Evans (9)]. Considerable quantities are present in the bee brain [Mercer et al. (22)], and radioactive OA was found to bind particularly strongly in the mushroom bodies [Erber et al. (6)].

Studies in flies [Long and Murdock (12)] and bees [Menzel et al. (19)] reveal that the OA agonists synephrine and the foramidines chlordimeform (CDF) and dichlordimeform (DCDF) stimulate feeding behavior and enhance the sensitization effect of sucrose stimulation. Groups of bees with relatively low learning performance learn better after feeding with CDF (Fig. 7).

Satiated animals which show neither the sucrose-induced PER nor any learning recover both the reflex and learning capacity after OA is injected into the hemolymph (Fig. 8). Local injections of small quantities of OA into the calyx or the α-lobe of the CP enhances memory formation (see Fig. 7). The retrieval from the

![Graph](image_url)

**Fig. 7.** The effect of chlordimeform (CDF) on olfactory conditioning in animals with relatively low learning performance. The animals were fed with sucrose solution (control group: “sucrose”, number of animals: 36) or sucrose solution containing $10^{-3}$ M or $10^{-4}$ M CDF (experimental group “CDF $10^{-3}$-sucrose”, number of animals: 28, and “CDF $10^{-4}$-sucrose, number of animals: 32) 16 h before the conditioning. The graph shows the respective acquisition functions. The data points are not significantly different on a point to point comparison ($\chi^2$-test), however, it is quite obvious that initial learning is enhanced with CDF in a dose dependent way. (This experiment was performed by students on the Neurobiology course at the Freie Universität Berlin 1989 under the supervision of R. Menzel.)
olfactory memory is enhanced if synephrine is applied to the calyx, but no significant effect was found after injection into the α-lobes. It appears that the facilitatory effect of OA or the OA-receptor agonist synephrine is neither specific with respect to the behavioral categories (feeding, sensitization, learning and memory retrieval) nor particularly specific for the localisation within the brain. It seems rather that OA receptors are likely to be involved in generally arousing networks.

Noradrenaline (NA)

The above conclusion on the action of OA also applies in general to NA, particularly when it is injected locally in small quantities and if the test animals have a relatively low learning rate. NA enhances the storage process. Retrieval after NA injection has not yet been studied (Fig. 9). Injections of large quantities of NA into the median protocerebrum of animals with high learning rate reduces memory formation [MENZEL et al. (19)]. The significance of the reversal of the NA-effects is presently not understood, but it is not uncommon in behavioral pharmacology that effects follow a U-shape function with a sign reversal [e.g.
Fig. 9. A summary of the effects of OA, NA and 5-HT when injected locally in small quantities (2–8 nl) in each of the paired structures of the mushroom bodies. Two series of experiments were performed, the storage test (injections before conditioning) and the retrieval test (injections after conditioning). Arrows pointing upwards indicate an enhancement of the storage or retrieval, arrows pointing downwards a reduction of storage or retrieval (see text for explanation). [After Bicker and Menzel (1).]

DUNN (5)]. Nevertheless, the results collected so far show the importance of an adrenergic (or closely related) mechanism in memory formation.

The overlap in the pharmacological profile between α-adrenergic receptors (as determined in vertebrates) and octopaminergic OA2-receptors [as determined in insects, Evans (9)] makes it very difficult to characterise more closely the receptor type responsible for the facilitatory effects after local injections of OA or NA. Synephrine, OA and NA act similarly. Phentolamine, an OA2-receptor and alpha-adrenergic antagonist, inhibit memory formation. Since clonidine and yohimbine have no significant effects, it is likely that an OA2-receptor mechanism in the mushroom bodies rather than an alpha-adrenergic or OA1-receptor mechanism is responsible for the improvement of memory storage and retrieval [Michelsen (23)]. The suspected OA2-receptor mechanism in the mushroom bodies is unlikely to be involved in a general arousing system, since OA, synephrine and phentolamine have no significant effects on the sensory and motor components of the reflex if the substances are injected into the mushroom body. Thus, the generally
arousing network, also comprising an OA2-receptor system, is probably located outside the mushroom bodies.

**Serotonin (5-HT)**

5-HT antagonises the action of OA and NA (see Fig. 9). Large or small amounts of 5-HT injections consistently reduce memory storage and retrieval. Furthermore, no evidence was found on whether the animals were particularly good or poor learners. Very little is known so far on the pharmacological profile of the 5-HT effects and whether behavioral components unrelated to olfactory learning and memory are also affected.

**Is cAMP involved as a second messenger?**

Neuromodulators exert their actions through second messengers such as cAMP, and indeed OA, NA and DA have a strong stimulatory effect on the adenylate cyclase activity in in-vitro incubation tests with bee brain tissue. In the purified membrane fraction OA stimulates and 5-HT inhibits adenylate cyclase activity [SUGAWA et al. (26, 27)]. Is it possible to identify such a second messenger with behavioral test procedures? The drug forskolin is known to penetrate easily into cell membranes and to stimulate cAMP synthesis. In the crude homogenates of bee brain forskolin causes a 16-fold increase in cAMP synthesis. We, therefore, injected various doses of forskolin into the median protocerebrum and ran storage and retrieval tests (Fig. 10). Retrieval of olfactory memory is significantly increased for a period of about 15 min after injection. Memory formation, however, is only slightly and not significantly increased. The results of these kind of tests were quite variable, and it appeared that the positive forskolin effect in the retrieval test was less reliable in groups of animals which learned particularly well.

In a separate series of experiments animals with very low learning rates were injected with dibutyrly-cAMP, a stable and active analog of cAMP (Fig. 11). Here, the acquisition of an olfactory CS was significantly improved when compared with two different control groups. These results indicate that cAMP may indeed be involved in the cascade of modulatory processes related to memory formation and memory retrieval. It became also clear that the level of cAMP is a limiting factor only under certain conditions, e.g. particularly low learning rate. Therefore, the manipulation of cAMP concentration leads to behaviorally detectable consequences only under such conditions.

We next asked whether the in-vivo synthesis of cAMP in the bee brain is related to behavioral measures of learning and behavior. It was first found that the cAMP-
Fig. 10. The effect of forskolin on the retrieval (a) and storage (b) of olfactory memory. a: In the retrieval test about 30 nl $10^{-4}$ forskolin was injected via the median ocellar nerve into the median protocerebrum after two conditioning trials. The animals were tested 5, 15, 30 and 60 min later, and a significantly higher response rate was found for the first two tests (stars, $p \leq 0.01$ $\chi^2$-test). b: In the storage test the animals were injected with $10^{-3}$ M or $10^{-4}$ M forskolin as described above, but 5 min before a single learning trial. The 4 tests T1–T4 reveal that all response rates were somewhat higher than in the control group, but these differences are not statistically significant.
Fig. 11. The effect of the cAMP analog dibutyryl-cAMP on the acquisition of olfactory memory was tested at a time when learning performance was low. Three groups of animals were studied (total number of animals, n = 58); Ringer control = 10 nl, Ringer was injected into the median protocerebrum prior to conditioning; 5% ethanol control = 10 nl of 5% ethanol in ringer was injected; experimental group = 10 nl of 10^-4 M dibutyryl cAMP dissolved in 5% ethanol-ringer was injected. The single learning trial followed 3 min after injection, and the animals were tested 4 times (T1-T4) at the indicated time intervals. The ordinate gives the response rate to the conditioned stimulus alone.

[Modified from Sugawa et al. (26, 27)].

Synthesis was about 2 times higher in the mushroom bodies than in the remaining protocerebrum. When animals were tested which were exposed to one conditioning trial and subsequently 3 test trials, a significant difference was found in the enzyme activity of the cAMP cascade in the mushroom bodies, but not in the rest of the protocerebrum: more cAMP was synthesized in the mushroom bodies of those animals which did not perform the conditioned response in the test trials [Sugawa et al. (26, 27)].

Conclusion

Neuropharmacological experiments with the honey bee have been carried out to address the question as to which biogenic amine might be involved in learning and memory and whether cAMP is involved as an essential second messenger. The
honey bee is particularly well suited for such experiments, simply because it
changes its behavior quickly and effectively by learning processes. Olfactory
learning is particularly fast and stable, and a long lasting memory is produced after
a single pairing of an odor with a sucrose reward even under restrained conditions
and with the brain exposed. The animal extends its proboscis as a conditioned
response (CR) to the presentation of an initially neutral odor stimulus. This
learning process can be manipulated by global or local injections of small quantities
of substances directly into the brain. The effects of a drug on the processes involved
in memory formation and those in the retrieval of memory can be separated,
because a single learning trial lasts only a few seconds, and the immediate drug
effects are fully reversible. The action of a drug on memory formation is tested by a
procedure called the “storage test”, and that on memory retrieval by the “retrieval
test”. For example, injection of DA (10^{-6} M) into the brain after a conditioning
trial (retrieval test) induces a transient reduction of the conditioned response. If
DA is injected before conditioning and the animal is conditioned at a time of
optimal action of DA (storage test), learning is not impaired. Thus, DA interferes
selectively with the retrieval process but not with that of olfactory coding, motor
response or memory storage. If the storage test is performed with an amine, which
interferes with the storage process, the drug effect is not reversible, but lasts for as
long as the animals are tested. Since the motor performance during the optimal
action of any test substance is not altered and all animals learn equally well as
controls at a later time, one can conclude that indeed the test substances interfere
with the memory processes and not with the sensory or motor components of the
task.

The analysis of local injections of biogenic amines reveals a neuropil pattern of
action which depends on the transmitter and the behavioral components tested.
Most importantly, learning and retrieval can be improved or depressed depending
on the amines used. OA mimicks the sensitizing action of the US (sucrose stimulus)
and stimulates feeding behavior when its is injected into the antennal (AL) and
dorsal lobe (DL). If OA is injected into the pendunculus of the mushroom bodies
close to the input synapses of the chemosensory relay neurons, no changes are
found for the reflex components but both components of learning, namely memory
formation and retrieval, are enhanced. OA has no effect when injected into the
output regions of the mushroom bodies, the alpha-lobes, whereas NA injections
into this region enhance memory formation particularly in animals which have
lower learning rates. 5-HT on the other hand antagonises the facilitatory actions of
OA and NA in the mushroom bodies while leaving the nonassociative modulation
of the reflex unaffected. All these results support the conclusion that the
deutocerebral sensory input regions of the bee brain are responsible for the
nonassociative modulation of the reflex, whereas the mushroom bodies are
Working hypothesis:

\[
\begin{align*}
\text{OA/NA} & \quad \text{modulated} & \quad \text{cAMP:} \\
\text{5-HT} & \quad \text{activity} & \quad \text{motivation arousal} \\
\end{align*}
\]

(high \quad \text{US-only, CS-only effects})

(low \quad \text{pairing effects (learning), unattentiveness, satiation})

Fig. 12. A schematic diagram of the working hypothesis (see text for details).

specifically involved in the long-term formation and read-out of associative memory.

The distinction between a storage and retrieval mechanism implies that the location of the memory trace is not the main sensory pathway between antennal lobe and SOG, because this pathway remains functional during the pharmacological block of the conditioned response. Anatomical studies of the insect brain have indeed revealed prominent fiber tracts which leave the main sensory pathway and enter the mushroom body neuropil (e.g. the mAGT shown in Fig. 2).

The aminergic modulations are likely to utilize cAMP as an important second messenger. Fig. 12 summarizes the results reported here and illustrates our current working hypothesis for memory formation in the honey bee. A key function is the up and down regulation of the adenylate cyclase which is mediated by the antagonising modulatory systems OA/NA and 5-HT. Specific pairing effects (learning) are only possible within the range of modulated cAMP levels. High cAMP levels are indicative for high motivation and arousal, low cAMP levels for unattentiveness and satiation after full feeding. Such a model calls for additional studies including those on the role of cAMP degradation by diesterases and phosphatases.

Acknowledgements

Supported by the Deutsche Forschungsgesellschaft. We are grateful to Dr. A. Mercer, Dr. G. Becker and G. Braun for allowing us to report unpublished results (see Fig. 4). The students of the Neurobiology course 1989 at the Freie Universität Berlin contributed to the recent results reported here. We are particularly grateful to Dr. P. Stevenson for his help with the English. The Hoechst AG provided us with a free sample of Forskolin. We are particularly grateful to Dr. Hock, Dr. Schorr and Prof. Scholtholt.
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