

Research

Cyclic nucleotide-gated channels, calmodulin, adenylyl cyclase, and calcium/calmodulin-dependent protein kinase II are required for late, but not early, long-term memory formation in the honeybee

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Memory is a dynamic process that allows encoding, storage, and retrieval of information acquired through individual experience. In the honeybee *Apis mellifera*, olfactory conditioning of the proboscis extension response (PER) has shown that besides short-term memory (STM) and mid-term memory (MTM), two phases of long-term memory (LTM) are formed upon multiple-trial conditioning: an early phase (e-LTM) which depends on translation from already available mRNA, and a late phase (l-LTM) which requires de novo transcription and translation. Here we combined olfactory PER conditioning and neuropharmacological inhibition and studied the involvement of the NO-cGMP pathway, and of specific molecules, such as cyclic nucleotide-gated channels (CNG), calmodulin (CaM), adenylyl cyclase (AC), and Ca²⁺/calmodulin-dependent protein kinase (CaMKII), in the formation of olfactory LTM in bees. We show that in addition to NO-cGMP and cAMP-PKA, CNG channels, CaM, AC, and CaMKII also participate in the formation of a l-LTM (72-h post-conditioning) that is specific for the learned odor. Importantly, the same molecules are dispensable for olfactory learning and for the formation of both MTM (in the minute and hour range) and e-LTM (24-h post-conditioning), thus suggesting that the signaling pathways leading to l-LTM or e-LTM involve different molecular actors.

Learning leads to cellular and molecular changes in the nervous system, which constitute the basis of memory, the capacity to encode, store, and retrieve information acquired through individual experience. Memory is a dynamic process organized in at least two different forms, short-term memory (STM) and long-term memory (LTM), exhibiting different temporal courses and distinct underlying molecular processes (Kandel 2001). Studies on both vertebrates and invertebrates (Bartsch et al. 1995; Yin and Tully 1996; Abel et al. 1997) showed that LTM formation requires an increase of the intracellular concentration of 3',5'-cyclic adenosine monophosphate (cAMP) and the recruitment of cAMP-dependent protein kinase (PKA) that phosphorylates the transcription factor cAMP-responsive element-binding protein (CREB). Nitric oxide (NO)-cGMP (3',5'-cyclic guanosine monophosphate) signaling is also involved in transcription-dependent plasticity, both in vertebrates (Lu et al. 1999) and invertebrates (Lewin and Walters 1999; Müller 2000; Kemenes et al. 2002). A link between the NO-cGMP system and CREB activation during transcription-dependent long-term potentiation (LTP) has been found in mice (Lu et al. 1999).

Insect models have largely contributed to our understanding of the molecular underpinnings of memory (e.g., Menzel 1999; Davis 2005; Margulies et al. 2005; Eisenhardt 2006; Schwärzel and Müller 2006; Busto et al. 2010). Among insects, the honeybee *Apis mellifera* has played an influential role in the study of memory as it provides both behavioral access to learning and memory in controlled laboratory protocols and invasive techniques that can trace behavioral plasticity to cellular and molecular levels (Menzel 1999, 2001; Giurfa 2007; Giurfa and Sandoz 2012). In a laboratory protocol termed the “olfactory conditioning of the proboscis extension reflex,” harnessed honeybees learn to associate odorants with a sucrose reward (Takeda 1961; Bitterman et al. 1983; Giurfa and Sandoz 2012). When the antennae of a hungry, harnessed bee are touched with sucrose solution, the animal reflexively extends its proboscis to reach out to and suck the sucrose (proboscis extension reflex or PER). Neutral odorants blown to the antennae do not release such a reflex in naive animals. However, if an odorant is presented immediately before sucrose solution (forward pairing), an association is formed which enables the odorant to release the PER in a following test. This effect is clearly associative and constitutes a case of classical conditioning (Bitterman et al. 1983), i.e., the odorant acts as a conditioned stimulus

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(CS) and the sucrose solution as a rewarding, unconditioned stimulus (US).

One-trial conditioning induces a mid-term memory (MTM) that can be retrieved from minutes to ~24 h after conditioning, and to an early-LTM (e-LTM) that can be retrieved from 24 to 48 h after conditioning. Multiple-trial conditioning also leads to MTM and e-LTM but induces, in addition, a late LTM (l-LTM) that can be retrieved three or more days after conditioning (Menzel 1999; Eisenhardt 2006; Schwärzel and Müller 2006). While e-LTM depends on translation from already available mRNA, l-LTM requires de novo transcription and translation (Menzel 1999; Schwärzel and Müller 2006).

Multiple-trial conditioning, which induces e- and l-LTM, promotes prolonged PKA activation mediated by NO, while one-trial conditioning, which induces only e-LTM, promotes transient PKA activation that is not mediated by NO signaling (Müller 2000). How precisely cAMP and NO-cGMP pathways are linked to each other in LTM formation, and if and how they mediate different memories, remains unclear. A possible link has been suggested in the cricket *Gryllus bimaculatus*, where NO-cGMP signaling was proposed to activate cAMP-mediated signaling via cyclic nucleotide-gated cation channels (CNG channels) and Ca²⁺/calmodulin (CaM), thereby leading to LTM (Matsumoto et al. 2006). Here we studied the implication of these molecules and signaling pathways in l-LTM formation in the bee, taking advantage of the olfactory conditioning of PER. We also determined whether activations of adenylyl cyclases (ACs), which have been proposed as coincidence detectors integrating CS and US inputs, thereby facilitating associative learning and memory (Anholt 1994; Gervasi et al. 2010), and of the Ca²⁺/CaM-dependent protein kinase II (CaMKII) are crucial events underlying LTM formation, as found both in vertebrates (Anholt 1994; Makhinson et al. 1999; Wang et al. 2004; Shan et al. 2008) and other invertebrates (Akmal et al. 2010; Gervasi et al. 2010; Chen et al. 2012; Malik et al. 2013).

We conditioned and tested 6991 honeybees, thereby providing the most extensive data set ever produced in experiments on PER conditioning. We determined the effect of antagonists of NO synthase (NOS), sGC, CNG channels, CaM, CaMKII, and AC on olfactory l-LTM measured 3 d (72 h) after conditioning. We also determined the specificity of these molecules for l-LTM by comparing the effects of their pharmacological antagonists 3 h (MTM), 24 h (e-LTM), and 72 h after conditioning. We show that these molecules are required for odor-specific l-LTM but are neither necessary for earlier memory phases nor for learning.

Results

Experiment 1: Noninjected bees learn odor-sucrose associations and exhibit significant memory retention 3 d after conditioning

We first verified that noninjected bees exhibit fast appetitive olfactory learning and robust memory (LTM) retention 3 d after conditioning. Bees were conditioned either with 1-nonanol ($n = 74$) or 2-hexanol ($n = 74$). In both groups, acquisition and LTM retention performances were similar (ANOVA for repeated measurements: factor group, acquisition, $F_{(1,146)} = 1.16$, $P = 0.28$; retention, $F_{(1,146)} = 0.88$, $P = 0.35$; group \times trial interaction, acquisition, $F_{(2,292)} = 0.02$, $P = 0.98$; retention, $F_{(1,146)} = 0.03$, $P = 0.87$) so that data of these groups were pooled. In all further experiments, no differences between bees conditioned with 1-nonanol or 2-hexanol were detected ($P > 0.05$ in all cases). Data were, therefore, pooled and presented as a single group.

Pooled acquisition and retention performances of noninjected bees exhibited the typical response patterns observed in olfactory PER conditioning (see Fig. 1). Acquisition was fast and

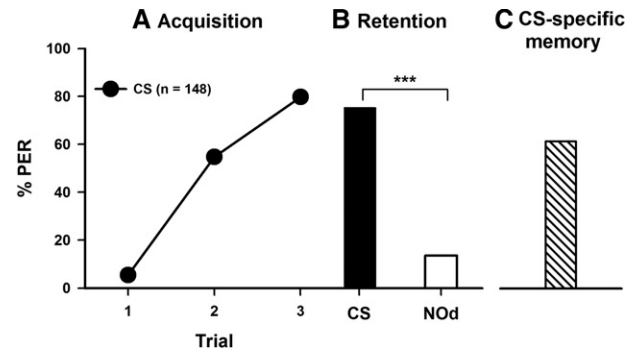


Figure 1. Olfactory acquisition and 3-d memory retention in honeybees. Bees were conditioned in three trials in which either 1-nonanol or 2-hexanol was used as conditioned stimulus (CS) paired with sucrose solution. (A) Acquisition was similar and effective in bees trained with these two odorants. (B) Three days after training, both groups were tested both with the CS and with a novel odorant (NOD) (1-nonanol for bees trained with 2-hexanol and vice versa). Bees responded to the CS and showed low generalization to the novel odor. Responses to the CS and to the novel odor were identical in both groups. (C) Percentage of bees exhibiting CS-specific memory, i.e., responding to the CS and not to the NOD during retention 3 d after conditioning. (***) $P < 0.001$.

highly significant ($F_{(2,294)} = 183.65$, $P < 0.00001$), reaching 80% of conditioned responses at the last acquisition trial (Fig. 1A). Three days after conditioning, bees exhibited robust LTM retention (Fig. 1B) evinced by highly significant responses to the CS (75% conditioned responses) and low generalization responses to the NOD (13.5%). Responses to the CS were significantly higher than those to the NOD (McNemar test, $\chi^2 = 89.01$, $df = 1$, $P < 0.00001$). CS-specific retention, defined as the proportion of bees which correctly responded to the CS and not to the NOD, was 61.5% (Fig. 1C).

Thus, noninjected bees learned the odorants efficiently and showed significant l-LTM retention 3 d after conditioning.

Experiment 2: Inhibition of protein synthesis and NO-cGMP signaling impairs l-LTM retention

Previous studies have demonstrated that NO-cGMP signaling and protein synthesis are involved in LTM formation in honeybees (Müller 1996, 2000; Wüstenberg et al. 1998; Schwärzel and Müller 2006). We thus verified that in our experimental conditions blocking the NO-cGMP signaling cascade and protein synthesis impairs LTM retention 3 d after conditioning.

NO-mediated activation of sGC leads to the synthesis of the second messenger cGMP. We blocked the NO-cGMP signaling pathway via injection of L-NAME (NOS-inhibitor, $n = 68$) or ODQ (sGC inhibitor, $n = 74$) and protein synthesis via injection of CHX ($n = 69$). Here and in the following experiments, injections were performed into the hemolymph of the thorax and, unless explicitly stated otherwise, 20 min before conditioning.

The doses of L-NAME and ODQ were chosen according to previous works in which these drugs acted as efficient NOS and sGC inhibitors in the honeybee nervous system (Müller 1996, 2000). The dose of CHX was three times larger than that reported as ineffective to block memory in bees (Table 1; Wittstock et al. 1993). None of the concentrations used induced significant (>5%) mortality in bees. Two control groups were injected with saline ($n = 70$) or saline containing 0.1% DMSO (D saline, $n = 62$).

Figure 2A shows the acquisition performance of the different groups. The two saline groups (saline and D saline) did not differ significantly from each other ($F_{(1,130)} = 0.39$, $P = 0.54$) and exhibited a nonsignificant group \times trial interaction ($F_{(2,260)} = 0.30$, $P =$

Table 1. Drugs and doses used in the present study

Drug	Target	Concentration (mM)	Dose (mg/kg)	Figure
L-NAME (L-NG-nitroarginine methyl ester)	NOS inhibitor	0.5	1.3	Figure 2
ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one)	sGC inhibitor	0.5	0.9	Figure 2
CHX (cycloheximide or 4-[(2R)-2-[(1S,3S,5S)-3,5-dimethyl-2-oxocyclohexyl]-2-hydroxyethyl]piperidine-2,6-dione)	Protein synthesis inhibitor	50	140.7	Figure 2
L-DIL (L- <i>cis</i> -diltiazem hydrochloride)	GNG channel inhibitor	0.2 0.5 1.0	0.9 2.3 4.5	Figure 3 Figure 3 Figures 3, 7, 8
W-7 (N-[6-aminoethyl]-5-chloro-1-naphthalenesulfonamide hydrochloride)	CaM antagonist	0.2 0.5 1.0	0.8 1.9 3.8	Figure 4 Figure 4 Figures 4, 7, 8
TFP (trifluoperazine or 10-[3-(4-methyl-1-piperazinyl)propyl]-2-[trifluoromethyl]-10H-phenothiazine dihydrochloride)	CaM antagonist	0.5 5.0	1.9 19.4	Figure 4 Figure 4
R24571 (calmidazolium or 3-[bis(4-chlorophenyl)methyl]-1-[2-(2,4-dichlorophenyl)-2-(2,4-dichlorophenyl)methoxy]ethyl]-1H-imidazolium, monochloride)	CaM antagonist	0.5 5.0	3.4 34.4	Figure 4 Figure 4
KN-62 (1-[N,O-bis-(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine)	CaMKII inhibitor	0.5 2.0	3.6 14.4	Figure 5 Figures 5, 7, 8
DDA (2',5'-dideoxyadenosine)	Adenylyl cyclase inhibitor	0.5 1.0 5.0	1.2 5.9 11.8	Figure 6 Figure 6 Figures 6, 7, 8
SQ 22536 (9-[tetrahydrofuryl]-adenine)	Adenylyl cyclase inhibitor	0.1	0.1	Figures 6, 7
MDL 12330A (<i>cis</i> -N-[2-phenylcyclopentyl]-azacyclotridec-1-en-2-amine)	Adenylyl cyclase inhibitor	0.1	0.2	Figures 6, 7

0.74) so that their results were pooled and presented as a single curve ("saline," $n = 132$) (Fig. 2A). Acquisition did not differ among all four groups ($F_{(3,373)} = 0.20, P = 0.90$) and the interaction was also not significant ($F_{(6,746)} = 0.22, P = 0.97$), showing that all bees learned to respond to the CS in a similar way. Thus, none of the three drugs (L-NAME, ODQ, and CHX) affected acquisition.

In the 3-d retention tests, the two saline groups (saline and D saline) did not differ significantly from each other (CS and NOD responses, $F_{(1,130)} = 0.32, P = 0.58$) and exhibited a nonsignificant interaction ($F_{(1,130)} = 0.20, P = 0.65$) so that their results were pooled again and presented as a single saline group ("saline," $n = 132$) (Fig. 2B). Bees in this control group responded significantly more to the CS than to the NOD ($\chi^2 = 75.01, df = 1, P < 0.00001$) and exhibited a high CS-specific memory (57.7%). Bees injected with CHX, L-NAME, or ODQ showed fewer conditioned responses (between 25.7% and 36.2%) than the control group, inducing a significant heterogeneity in retention performance among the four groups (CS responses, $F_{(3,339)} = 10.93, P < 0.0001$). This heterogeneity was due to the fact that CS responses of the control group were significantly higher than those of the drug-injected groups (Tukey test, $P < 0.05$ for all comparisons), thus indicating that these drugs induced a similar impairment of 3-d CS retention. CS responses of the CHX, L-NAME, and ODQ groups were statistically indistinguishable ($P > 0.50$ for all comparisons). Responses to the NOD were equally low in all groups

including saline bees (NOD responses, $F_{(3,339)} = 0.56, P = 0.65$). Although reduced, CS responses were significantly higher than NOD responses in all drug-injected groups (L-NAME, $\chi^2 = 13.47, df = 1, P < 0.0005$; ODQ, $\chi^2 = 12.50, df = 1, P < 0.0005$; CHX, $\chi^2 = 15.43, df = 1, P < 0.0002$), thus indicating that CS retrieval was still possible. Indeed, CS-specific memory was 28.99%, 26.47%, and 22.97% for CHX-, L-NAME-, and ODQ-injected bees, respectively (Fig. 2C). CS-specific memory differed between

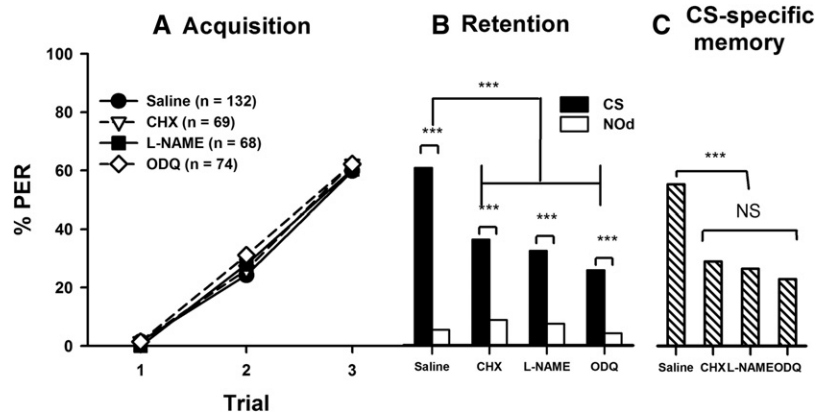


Figure 2. Inhibition of translation and of the NO-cGMP pathway impairs 3-d retention. Twenty minutes before conditioning, bees received an injection (1 μ L) of either saline solution (saline), the protein synthesis-inhibitor CHX (50 mM), the NOS-inhibitor L-NAME (0.5 mM), or the sGC-inhibitor ODQ (0.5 mM). Bees were conditioned either with 1-nonanol or with 2-hexanol; performances of both subgroups did not differ significantly and their data were pooled. (A) All groups showed similar and effective acquisition. (B) In the 3-d retention test, all drug-injected groups were significantly impaired in their CS response (black bars) when compared to the saline group (control). Yet, they still exhibited a CS-specific memory as responses to the novel odorant (NOD, white bars) were significantly lower in all groups. Performance of all three drug-injected groups (CHX, L-NAME, ODQ) was equivalent. (C) Percentage of bees exhibiting CS-specific memory, i.e., responding to the CS and not to the NOD during retention 3 d after conditioning. (***) $P < 0.001$, (NS) nonsignificant.

saline-injected and drug-injected bees (Tukey test for multiple comparisons between proportions, $q_{\infty,4} > 3.633$ for all three comparisons, $P < 0.001$) but not between drug-injected bees ($q_{\infty,4} > 3.633$ for all three comparisons, NS).

Thus, inhibition of protein synthesis via CHX and of NO-cGMP signaling via L-NAME and ODQ impairs I-LTM retention in honeybees.

Experiment 3: Inhibition of CNG channels impairs I-LTM retention

CNG channels are Ca^{2+} -permeable channels activated by cAMP and/or cGMP. They are crucial for sensory transduction at the level of olfactory receptors (Broillet and Firestein 1996) and for discrimination of olfactory stimuli in the presence of an adapting background odor in mammals (Kelliher et al. 2003). Their involvement in olfactory learning and memory has been postulated in crickets (Matsumoto et al. 2006), but has not been studied so far in any other insect model. We thus examined the effect of L-DIL, a CNG channel inhibitor (Kaupp and Seifert 2002), on olfactory learning and memory in honeybees (Fig. 3). Bees were injected either with saline ($n = 62$) or with L-DIL delivered at three different concentrations, 0.2 mM ($n = 57$), 0.5 mM ($n = 64$), or 1 mM ($n = 59$) (Table 1).

All four groups exhibited similar acquisition performances ($F_{(3,238)} = 0.65$, $P = 0.59$; interaction, $F_{(6,476)} = 0.30$, $P = 0.94$) and reached 56.5%–67.2% of conditioned responses at the third trial (Fig. 3A), thus showing that L-DIL injection did not affect acquisition. Retention performances varied depending on treatment (ANOVA performed on CS and NOd responses, $F_{(3,238)} = 3.84$, $P < 0.02$) (Fig. 3B). CS responses decreased significantly from 54.8% in the saline group to 18.6% in the group injected with 1 mM L-DIL ($F_{(3,238)} = 7.61$, $P < 0.0001$). CS responses did not differ between the saline group and the group injected with the lowest L-DIL dose (0.2 mM; Tukey test, $P = 0.32$), but they were significantly different between the saline group and the groups injected with the intermediate and the highest L-DIL doses (L-DIL 0.5 and 1 mM, $P = 0.006$ and $P < 0.0001$, respectively). A

dose-dependent drug effect was found as the highest L-DIL dose (1 mM) induced the lowest level of CS responses while the lowest L-DIL dose induced the highest level of CS responses ($P < 0.05$); CS responses of the 0.5 mM L-DIL group were at an intermediate level. By contrast, responses to the novel odorant did not differ significantly between groups (NOd responses, $F_{(3,238)} = 1.33$, $P = 0.26$). Significant discrimination between the CS and the NOd was found in the saline group ($\chi^2 = 28.03$, $df = 1$, $P < 0.0001$) and in the group with the lowest (0.2 mM) L-DIL dose ($\chi^2 = 16.06$, $df = 1$, $P < 0.001$). Discrimination was nonsignificant for the groups injected with the intermediate (0.5 mM) dose ($\chi^2 = 3.50$, $df = 1$, $P = 0.06$) and the highest L-DIL (1 mM) dose ($\chi^2 = 3.13$, $df = 1$, $P = 0.08$). CS-specific memory was, respectively, 17.19% and 11.8% for these groups while it was 48.39% and 31.58% for the saline group and for the group with the lowest (0.2 mM) L-DIL dose (Fig. 3C). CS-specific memory differed between saline-injected and drug-injected bees ($q_{\infty,4} > 3.633$ for all three comparisons, $P < 0.001$) and also between bees injected with the lowest L-DIL dose and bees injected with the two other L-DIL doses ($q_{\infty,4} > 3.633$ for both comparisons, $P < 0.01$). No differences in CS-specific memory were found between bees injected with L-DIL 0.5 and 1 mM ($q_{\infty,4} < 3.633$, NS).

Thus, inhibition of CNG channels impairs I-LTM retention in honeybees.

Experiment 4: Inhibition of CaM impairs I-LTM retention

CaM is a major Ca^{2+} -binding protein found in the central nervous system. Although a large number of studies have investigated the effects of impairing enzymes targeted by CaM (e.g., NOS, CaMKII, MAPK) on learning and memory, there are relatively few reports about the effects of direct inhibition of CaM (Malenka et al. 1989; Nakazawa et al. 1995; Margrie et al. 1998; Limback-Stokin et al. 2004). We investigated the effect of CaM inhibition on olfactory learning and memory in honeybees using three structurally different CaM antagonists, W-7, TFP, and R24571, delivered at different concentrations (Fig. 4).

Bees were injected with saline or a CaM antagonist before conditioning. For all three CaM-antagonist injected groups, acquisition was similar and fast and did not differ from that of their corresponding control groups (W-7, $F_{(3,317)} = 0.48$, $P = 0.70$; TFP, $F_{(2,188)} = 1.11$, $P = 0.33$; R24571, $F_{(2,206)} = 0.90$, $P = 0.41$) (Fig. 4A,D,G). At the third conditioning trial, bees of all groups reached similar levels of conditioned responses (55.7%–69.4%). Thus, injection of the three different CaM antagonists did not affect acquisition.

Figure 4B shows 3-d retention performances after W-7 injection. There was a significant variation in retention levels among groups (CS and NOd responses, $F_{(3,317)} = 7.96$, $P < 0.0001$). The level of CS responses was significantly reduced in W-7 injected bees with respect to that of the saline group (CS responses, $F_{(3,317)} = 16.21$, $P < 0.00001$), but the three W-7 injected groups did not differ in their levels of CS responses (Tukey test, $P > 0.40$ for all comparisons). Response levels to the NOd did not vary among groups (NOd responses, $F_{(3,317)} =$

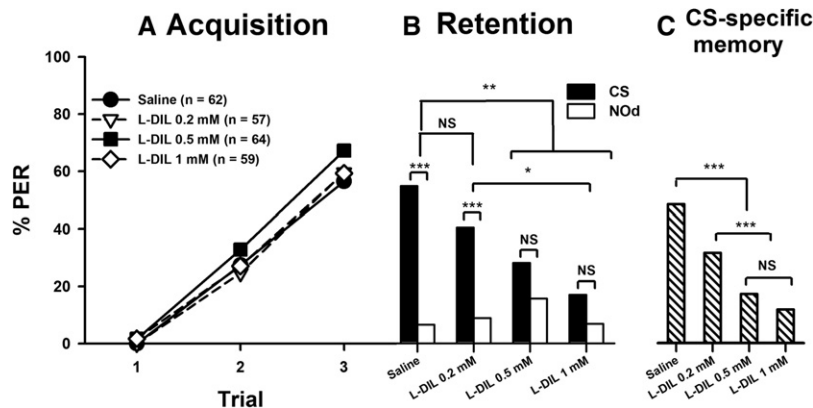


Figure 3. Inhibition of CNG channels impairs 3-d retention. Twenty minutes before conditioning, bees received an injection (1 μL) of either saline solution (saline) or one of three different concentrations (0.2 mM, 0.5 mM, or 1 mM) of the CNG-channel-inhibitor L-DIL. Bees were conditioned either with 1-nonanol or with 2-hexanol; performances of both subgroups did not differ significantly and their data were pooled. (A) All groups showed similar and effective acquisition. (B) In the 3-d retention test, the groups injected with the intermediate (0.5 mM) and the highest (1 mM) dose of L-DIL exhibited a significant decrease of CS responses with respect to the saline control. In these two groups, there was no significant difference between the response to the CS (black bars) and the response to the NOd (white bars), thus indicating a loss of the CS-specific memory. (C) Percentage of bees exhibiting CS-specific memory, i.e., responding to the CS and not to the NOd during retention 3 d after conditioning. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (NS) nonsignificant.

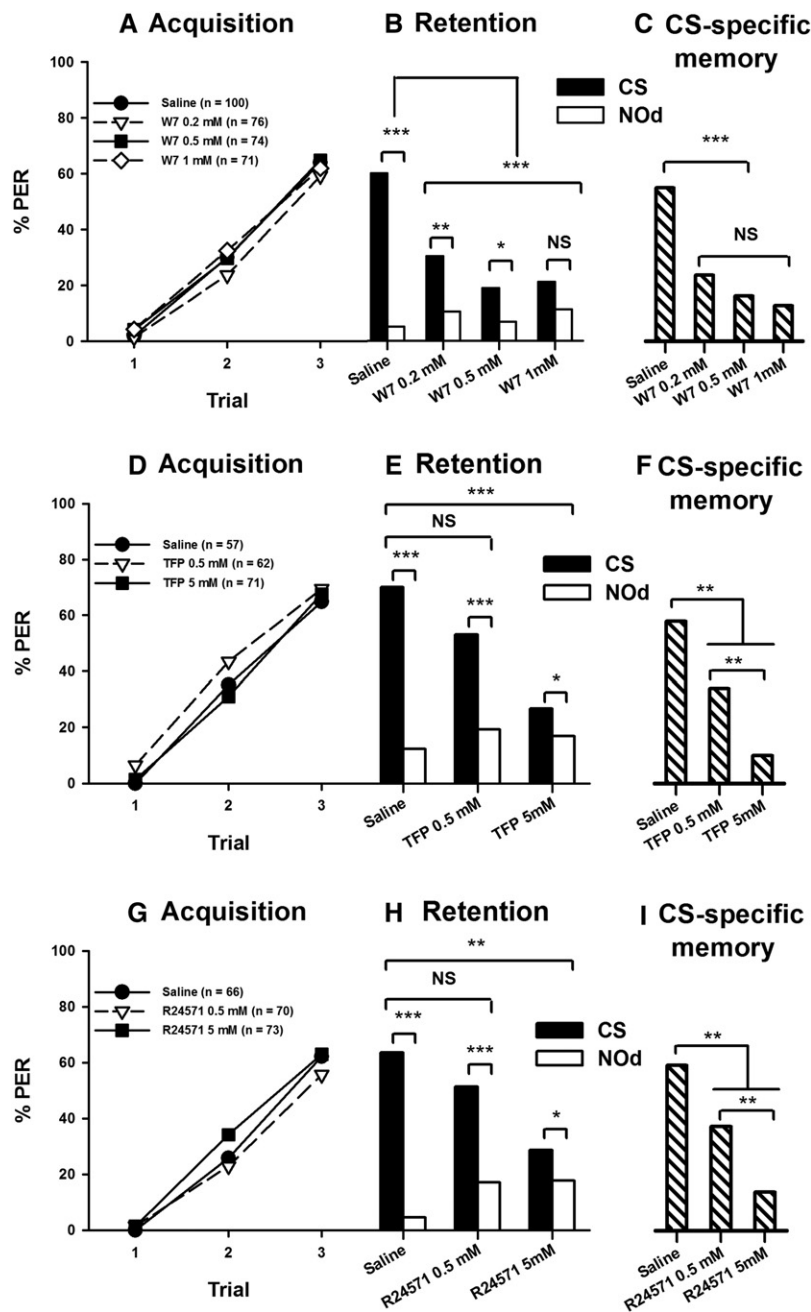


Figure 4. Inhibition of CaM impairs 3-d retention. Twenty minutes before conditioning, bees received an injection of either saline solution (saline) or one of two to three different concentrations of the CaM antagonists W-7 (A,B), TFP (C,D), or R24571 (E,F). Bees were conditioned either with 1-nonananol or with 2-hexanol; performances of both subgroups did not differ significantly and their data were pooled. (A,D,G) All groups showed similar and effective acquisition. (B,E,H) In the 3-d retention test, all groups injected with the highest drug doses showed a significant decrease of CS responses with respect to the saline control, as well as to those treated with lower doses of W-7. Drug effects were dose-dependent. In the group injected with 1 mM W-7, there was no significant difference between the responses to the CS (black bars) and to the NOd (white bars), indicating a loss of CS-specific memory. (C,F,I) Percentage of bees exhibiting CS-specific memory, i.e., responding to the CS and not to the NOd during retention 3 d after conditioning. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (NS) nonsignificant.

1.01, $P = 0.39$). The saline group responded significantly more to the CS than those to the NOd ($\chi^2 = 53.02$, $df = 1$, $P < 0.0001$). Discrimination was also significant in the groups injected with the lowest (0.2 mM) and the intermediate (0.5 mM) W-7 doses

($\chi^2 = 9.33$, $df = 1$, $P < 0.005$ and $\chi^2 = 4.27$, $df = 1$, $P < 0.05$, respectively) but not in the group injected with the highest dose (1 mM, $\chi^2 = 3.27$, $df = 1$, $P = 0.07$). The proportion of bees exhibiting CS-specific memory was 55% in the saline group and decreased to 23.68% (0.2 mM), 16.22% (0.5 mM), and 12.68% (1 mM) in the W-7 injected groups (Fig. 4C). CS-specific memory differed between saline-injected and drug-injected bees ($q_{\infty,4} > 3.633$ for all three comparisons, $P < 0.001$) but not between drug-injected bees ($q_{\infty,4} < 3.633$ for all three comparisons, NS).

Figure 4E shows 3-d retention performances after TFP injection. There was a significant variation in retention among groups (CS and NOd responses, $F_{(2,188)} = 4.74$, $P < 0.01$). Specifically, the level of CS responses varied significantly among groups (CS responses, $F_{(2,188)} = 13.33$, $P < 0.0001$). While the lower TFP dose (0.5 mM) did not induce any significant drop in CS responses compared to the control group (Tukey test, $P = 0.16$), the higher dose (5 mM) decreased CS responses, and rendered them significantly different both from those of the control group ($P < 0.0005$) and of the group injected with the lower TFP dose ($P < 0.005$). Responses to the NOd did not differ among groups (NOd responses, $F_{(2,188)} = 0.60$, $P = 0.55$). All groups significantly discriminated the CS from the NOd (saline, $\chi^2 = 31.03$, $df = 1$, $P < 0.0001$; TFP 0.5 mM, $\chi^2 = 19.05$, $df = 1$, $P < 0.0001$; TFP 5 mM, $\chi^2 = 5.15$, $df = 1$, $P < 0.05$), but the proportion of bees exhibiting CS-specific memory decreased with TFP dose (saline group, 57.90%; TFP 0.5 mM, 33.87%; TFP 5 mM, 9.86%) (Fig. 4F). CS-specific memory differed between saline-injected and drug-injected bees ($q_{\infty,3} > 3.314$ for both comparisons, $P < 0.01$) and also between bees injected with the lowest and the highest TFP doses ($q_{\infty,3} > 3.314$, $P < 0.01$).

Figure 4H shows 3-d retention performances after R24571 injection. There was no significant variation in retention among groups (CS and NOd responses, $F_{(2,206)} = 1.91$, $P = 0.15$). Yet, the level of CS responses varied significantly among groups (CS responses, $F_{(2,206)} = 5.82$, $P < 0.005$). The lower R24571 dose (0.5 mM) did not induce any significant drop in CS responses with respect to the control group (Tukey test, $P = 0.48$). In contrast, the higher dose (5 mM) decreased CS responses and rendered them significantly different from those of the saline group ($P < 0.005$) but not from those of the group injected with the lower R24571 dose ($P = 0.07$). Responses to the NOd differed among groups (NOd responses, $F_{(2,206)} = 3.32$, $P < 0.05$) as they increased

with the dose of R24571. All groups significantly discriminated the CS from the NOd (saline, $\chi^2 = 37.03$, $df = 1$, $P < 0.0001$; R24571 0.5 mM, $\chi^2 = 18.89$, $df = 1$, $P < 0.0001$; R24571 5 mM, $\chi^2 = 4.08$, $df = 1$, $P < 0.05$), but the proportion of bees exhibiting CS-specific memory decreased with increasing R24571 doses (saline group, 59.09%; R24571 0.5 mM, 37.14%; and R24571 5 mM, 13.70%) (Fig. 4I). CS-specific memory differed between saline-injected and drug-injected bees ($q_{\infty,3} > 3.314$ for both comparisons, $P < 0.01$) and also between bees injected with the lowest and the highest R24571 doses ($q_{\infty,3} > 3.314$, $P < 0.01$).

All in all, the three CaM antagonists significantly decreased 3-d retention by reducing responses to the CS when injected at a high dose.

Experiment 5: Inhibition of CaMKII impairs

I-LTM retention

The Ca^{2+} /CaM-dependent protein kinase II (CaMKII) has been linked to neuronal plasticity associated with long-term potentiation as well as with structural synaptic plasticity (Micheau and Riedel 1999). Previous work in adult honeybees has shown that the single CaMKII gene found in honeybees is strongly expressed in the mushroom bodies (MBs) (Kamikouchi et al. 2000), brain centers associated with sensory integration, learning, and memory formation (Giurfa 2007; Giurfa and Sandoz 2012). Moreover, the activated (phosphorylated) form of the protein, pCaMKII, is predominantly concentrated in these structures in the adult bee brain (Pasch et al. 2011). We studied the effect of KN-62, a CaMKII inhibitor (Enslin et al. 1994), on olfactory acquisition and 3-d retention in honeybees.

Before conditioning, bees were injected either with saline ($n = 65$) or with one of two different concentrations of KN-62, 0.5 mM ($n = 61$) or 2 mM ($n = 71$). Figure 5A shows that there was no significant variation in acquisition among groups ($F_{(2,194)} = 0.35$, $P = 0.71$). In all three groups acquisition was similar and fast and, irrespective of treatment, all bees reached a similar level of conditioned responses at the third conditioning trial (between 57.4% and 67.6%). Thus, injection of KN-62 did not affect acquisition.

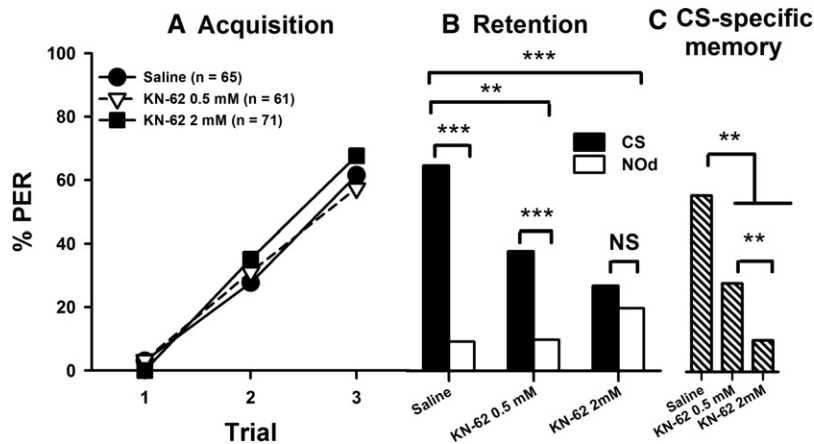


Figure 5. Inhibition of CaMKII impairs 3-d retention. Twenty minutes before conditioning, bees received an injection of either saline solution (saline) or one of two different concentrations (0.5 mM or 2 mM) of KN-62, a CaMKII-inhibitor. (A) All groups showed similar and effective acquisition. (B) In the 3-d retention test, the two groups injected with KN-62 showed a significant decrease of CS responses with respect to the saline control. In the group injected with 2 mM KN-62, there was no significant difference between the responses to the CS (black bars) and to the NOd (white bars), indicating a loss of CS-specific memory. (C) Percentage of bees exhibiting CS-specific memory, i.e., responding to the CS and not to the NOd during retention 3 d after conditioning. (**) $P < 0.01$, (***) $P < 0.001$, (NS) nonsignificant.

Figure 5B shows performances during the 3-d retention tests. There were significant differences among groups (CS and NOd responses, $F_{(2,194)} = 3.34$, $P < 0.05$). Specifically, groups differed significantly in their level of CS responses (CS responses, $F_{(2,194)} = 11.43$, $P < 0.0001$) as saline-injected bees responded more to the CS than the two groups injected with KN-62 (Tukey test, $P < 0.005$ in both cases); the two groups injected with the two doses of KN-62 did not differ from each other in their CS responses ($P = 0.38$). No significant variation in the groups' responses to the NOd were detected (NOd responses, $F_{(2,194)} = 2.08$, $P = 0.13$). While the control group and the group injected with the lower KN-62 dose exhibited significant discrimination between CS and NOd (saline, $\chi^2 = 34.03$, $df = 1$, $P < 0.0001$; KN-62 0.5 mM, $\chi^2 = 15.06$, $df = 1$, $P < 0.001$), the group injected with the higher KN-62 dose did not show any discrimination between these odorants ($\chi^2 = 1.78$, $df = 1$, $P = 0.18$). The proportion of bees that exhibited CS-specific memory decreased from 55.39% in the control group to 27.87% in the group injected with KN-62 0.5 mM, and to 9.86% in the group injected with KN-62 2 mM. CS-specific memory differed between saline-injected and drug-injected bees ($q_{\infty,3} > 3.314$ for both comparisons, $P < 0.01$) and also between bees injected with the lowest and the highest KN-62 doses ($q_{\infty,3} > 3.314$, $P < 0.01$).

Thus, inhibition of a CaMKII significantly impaired I-LTM by reducing CS-specific responses in a dose-dependent manner.

Experiment 6: Inhibition of AC impairs I-LTM

retention

Studies in a number of animal models have shown that the cAMP signaling pathway, including some adenylyl cyclases (AC), is crucial for the formation of LTM (Wong et al. 1999; Kandel 2001; Poser and Storm 2001). In honeybees, pharmacological inhibition of PKA, the primary target of cAMP, impairs LTM (Müller 2000). However, whether direct inhibition of AC affects honeybee learning and memory remains to be determined. In order to study the implication of AC in olfactory acquisition and 3-d retention, we injected bees 20 min before training with saline ($n = 100$) or with 2',5'-dideoxyadenosine (DDA), a P-site specific AC inhibitor (Bhattacharya et al. 2004). DDA was injected at three different concentrations, 0.5 mM ($n = 65$), 1 mM ($n = 66$), and 5 mM ($n = 69$).

Figure 6A shows that acquisition was fast and similar in all groups ($F_{(3,297)} = 0.09$, $P = 0.97$). At the third conditioning trial, bees reached a level of conditioned responses that was between 63.1% and 69.7%. Thus, injection of the AC inhibitor DDA did not impair olfactory acquisition.

The performances of the different groups in 3-d retention tests are shown in Figure 6B. Retention differed significantly among groups (CS and NOd responses, $F_{(3,297)} = 2.90$, $P < 0.05$); in particular, responses to the CS varied significantly (CS responses, $F_{(3,297)} = 5.99$, $P < 0.001$) as the saline group exhibited significantly more CS responses than all DDA-injected groups (Tukey test, $P < 0.05$ for all three comparisons). DDA-injected groups did not differ from each other in their CS responses ($P > 0.78$ for all three comparisons). Responses to the novel odorant were

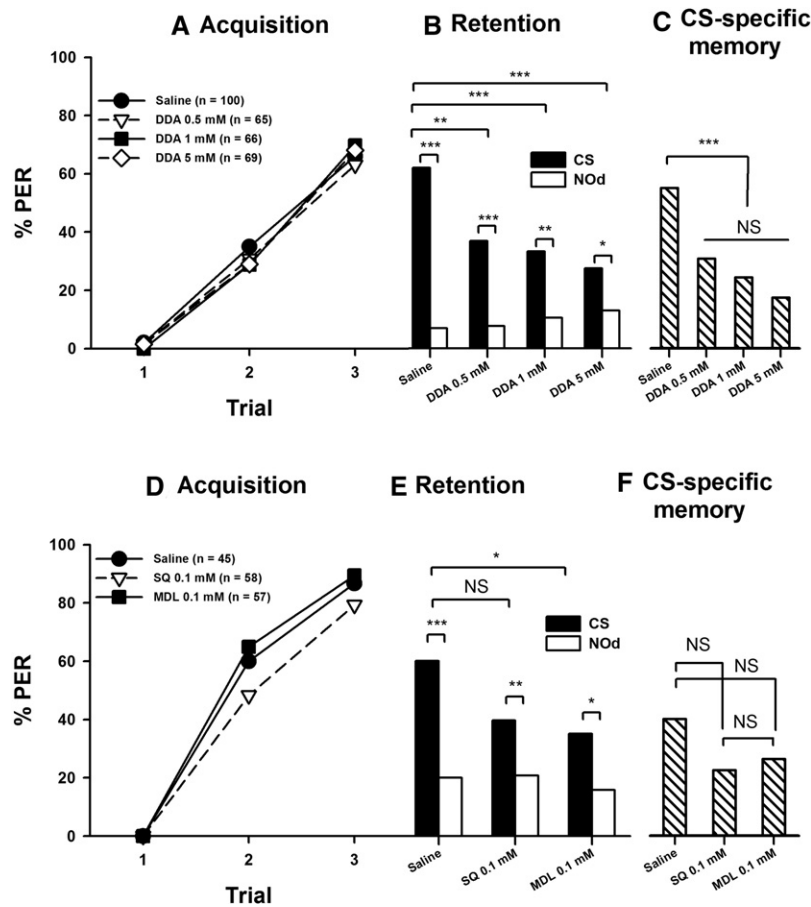


Figure 6. Inhibition of AC impairs 3-d retention. Twenty minutes before conditioning, bees were injected with either saline solution or with an AC antagonist. (A–C) Performance after injection of three different concentrations (0.5 mM, 1 mM, or 5 mM) of the AC-inhibitor 2',5'-dideoxyadenosine (DDA). (D–F) Performance after injection of the two AC inhibitors (0.1 mM each) SQ 22536 and MDL 12330A. (A) All groups showed similar and effective acquisition. (B) In the 3-d retention test, all DDA-injected groups showed a significant decrease of CS responses with respect to the saline control. Drug effects were dose-dependent. Although reduced, CS responses (black bars) were significantly higher than responses to the NOd (white bars) in all groups, thus showing that some CS-specific memory was preserved. (C) Percentage of bees exhibiting CS-specific memory, i.e., responding to the CS and not to the NOd during retention 3 d after conditioning. (D) All groups showed similar and effective acquisition. (E) In the 3-d retention test, CS responses (black bars) were significantly higher than responses to the NOd (white bars) in all groups. Yet, both AC antagonists induced a decrease of CS responses. This decrease was barely nonsignificant in 22536-injected bees ($P = 0.09$) and significant in MDL 12330A-injected bees. (F) Percentage of bees exhibiting CS-specific memory, i.e., responding to the CS and not to the NOd during retention 3 d after conditioning. Despite the decrease induced by the two AC antagonists, no significant differences were detected. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (NS) nonsignificant.

not affected by DDA injection (NOd responses, $F_{(3,297)} = 0.71$, $P = 0.54$).

Despite the significant decrease in CS responses in all three DDA groups, responses to the CS remained significantly higher than those to the NOd (saline, $\chi^2 = 53.02$, $df = 1$, $P < 0.0001$; DDA-62 0.5 mM, $\chi^2 = 15.43$, $df = 1$, $P < 0.001$; DDA-62 1 mM, $\chi^2 = 11.53$, $df = 1$, $P < 0.005$; DDA-62 5 mM, $\chi^2 = 5.79$, $df = 1$, $P < 0.02$). The proportion of bees exhibiting CS-specific memory (Fig. 6C) was 55.00% in the control group, and 30.77%, 24.24%, and 17.39% in the groups injected with DDA at concentrations of 0.5, 1, and 5 mM, respectively. CS-specific memory differed between saline-injected and DDA-injected bees ($q_{\infty,4} > 3.633$ for all three comparisons, $P < 0.001$) but not between DDA-injected bees ($q_{\infty,4} < 3.633$ for all three comparisons, NS).

These results show that inhibition of AC did not affect olfactory learning but decreased CS-specific responses 3 d after conditioning, without suppressing the capacity to discriminate the CS from the NO.

We verified this conclusion by performing a further experiment with two additional AC antagonists, SQ 22536 (Heinrich et al. 2001) and MDL 12330A (Gray et al. 1984), already assayed in other insect preparations. We injected bees with saline ($n = 45$) or with SQ 22536 ($n = 58$) or with MDL 12330A ($n = 57$) 20 min before acquisition. Both drugs were delivered at a concentration of 0.1 mM which was chosen based on preliminary experiments.

Figure 6D shows that all three groups of bees learned efficiently the odor–sucrose association ($F_{(2,150)} = 252.7$, $P < 0.0001$). At the third conditioning trial, they reached similar levels of conditioned responses, which varied between 81% and 89% ($F_{(2,140)} = 1.328$, $P = 0.268$). Thus, injection of two further AC inhibitors, SQ 22536 and MDL 12330A, failed again to impair olfactory acquisition.

The performances of the three groups in the 3-d retention tests are shown in Figure 6E. Although responses to the CS were significantly higher than those to the NOd in all three groups (saline, $\chi^2 = 16.06$, $df = 1$, $P < 0.001$; SQ 22536, $\chi^2 = 6.67$, $df = 1$, $P < 0.01$; MDL 12330A, $\chi^2 = 5.26$, $df = 1$, $P < 0.05$), they varied significantly between groups (CS responses, $F_{(2,157)} = 3.57$, $P < 0.05$). Despite the unusual decrease of CS responses from the third conditioning trial to the CS test in the saline group (compare with Fig. 6A,B), saline-injected bees exhibited significantly more CS responses than bees injected with MDL 12330A (Tukey test, $P < 0.05$). The CS response of the saline-injected bees did not differ from that of the SQ 22536-injected bees but was, nevertheless, close to significance ($P = 0.08$). SQ 22536- and MDL 12330A-injected bees did not differ from each other in their CS responses ($P = 0.87$). Responses to the NOd were similar in all three groups (NOd responses, $F_{(2,157)} = 0.10$, $P = 0.91$).

As in the previous experiment with DDA, the proportion of bees exhibiting CS-specific memory was higher in the control group (40%) than in the SQ 22536- (23%) and MDL 12330A-injected groups (26%). Yet, this difference did not reach significance ($q_{\infty,3} < 3.314$ for both comparisons, NS). No differences in specific memory were found between AC antagonist-injected groups ($q_{\infty,3} < 3.314$, NS).

Thus, three different AC antagonists yielded comparable results: none of them inhibited olfactory learning but they all affected 3-d retention; they induced a significant decrease of CS responses and a concomitant decrease of CS-specific retention,

which in the case of DDA was significant and in the case of SQ 22536 and MDL 12330A did not reach significance.

Experiment 7: Blocking of CNG-channel, CaM, CaMKII, and AC signaling impairs late-LTM but neither MTM nor early-LTM

The previous results indicate that the signaling pathways targeted by the different pharmacological agents assayed are involved in l-LTM formation. Whether they are also required for earlier memories remains to be determined. As none of the injected drugs affected olfactory acquisition, effects on STM in the minute range (i.e., corresponding to the ITI separating acquisition trials) can be excluded. We thus tested the effects of the drugs at intermediate delays to assess their possible impact on MTM (3 h) and e-LTM (24 h). Hence, bees were injected 20 min before conditioning with saline or with the highest tested dose of each drug (L-DIL 1 mM for CNG-channel signaling, W-7 1 mM for CaM signaling as it was the most effective CaM inhibitor, KN-62 2 mM for CaMKII signaling, and DDA 5 mM for AC signaling). We then compared retention performances at 3 h, 1 d, and 3 d by focusing on CS-specific memories resulting from each treatment.

Figure 7 shows the proportions of bees responding to the CS but not to the NOd (“CS-specific memory”) at the three retention delays (3 h, 1 d, 3 d) and for the four drugs assayed. The four drugs significantly reduced CS-specific l-LTM (3 d after conditioning) with respect to controls (Tukey test for multiple comparisons between proportions, $P < 0.001$ for all four 3-d comparisons) (Fig. 7A–D). However, they affected neither CS-specific MTM (3 h) nor CS-specific e-LTM (1 d) ($q_{\infty,6} < 4.03$, NS for all 3-h and 1-day comparisons) (Fig. 7A–D). Thus, blocking signaling through CNG channels, CaMKII, AC, or CaM signaling specifically blocks l-LTM but affects neither MTM nor e-LTM.

As AC has been suggested as a coincidence detector integrating CS and US inputs (e.g., Gervasi et al. 2010), the results of Figure 7D—but also of Figure 6, A and E—raise interrogations as blocking AC neither affected olfactory acquisition nor the early memory phases (MTM and e-LTM). To determine if this result was specific to the AC antagonist used (DDA), we repeated the experiments of Figure 7D using the two additional AC antagonists assayed in the previous experiment, SQ 22536 and MDL 12330A (Fig. 6D–F). Both were injected at a dose of 0.1 mM 20 min before acquisition. Retention performances were recorded 3 h and 1 d after conditioning, and compared to those observed at 3 d after conditioning.

Figure 7, E and F, shows the CS-specific memory at the three retention delays (3 h, 1 d, 3 d) for the saline group and the two antagonist-injected groups. Similarly to DDA, SQ 22536 and MDL 12330A neither impaired MTM (3 h after conditioning) nor e-LTM (1 d after conditioning) ($q_{\infty,3} < 3.314$, $P > 0.05$ for all comparisons).

Thus, irrespective of the AC antagonist used, AC was dispensable for acquisition and for early memory phases (STM, MTM, e-LTM).

Experiment 8: The effects of injection timing

To delimit the time window of sensitivity of l-LTM to some of the tested antagonists, we evaluated the effect of different injection times (Fig. 8) for the drugs L-DIL (1 mM), W-7 (1 mM), KN-62 (2 mM), and DDA (5 mM), all of which specifically impaired l-LTM at these doses when injected 20 min before conditioning (see Fig. 7). In this new experimental series, bees were injected either 90 min before conditioning (Fig. 8A) or 10 min after conditioning (Fig. 8B). In both cases they were tested for retention 3 d after conditioning. For the bees injected 90 min before conditioning,

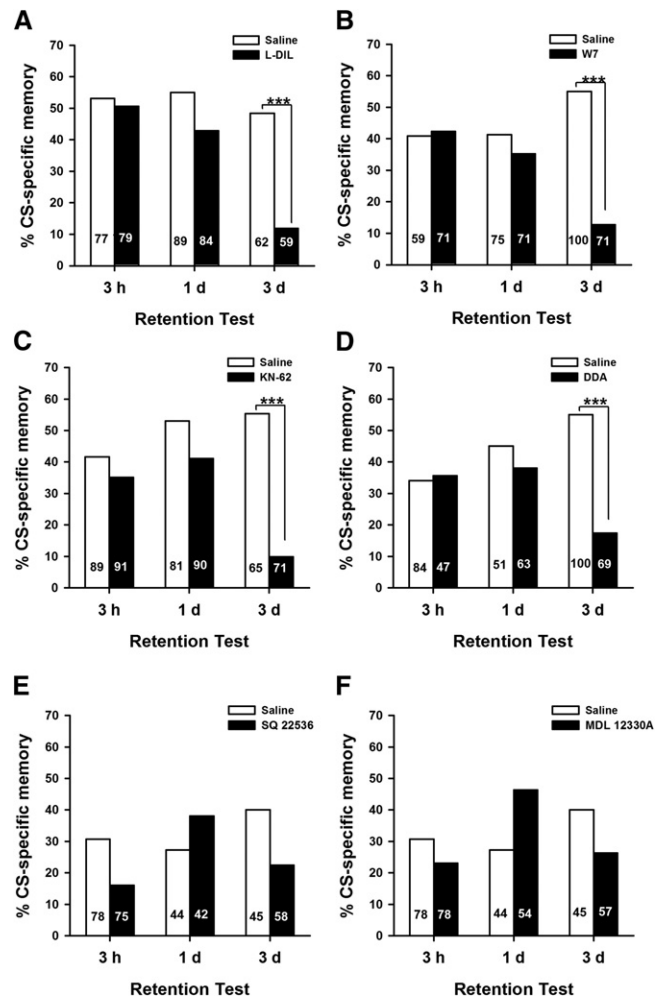


Figure 7. Dependence of CS-specific memory on testing period and injected drug. Twenty minutes before conditioning, animals were injected either with saline solution, or 1 mM L-DIL (A), 1 mM W-7 (B), 2 mM KN-62 (C), 5 mM DDA (D), 0.1 mM SQ 22536 (E), or 0.1 mM MDL 12330A (F). Retention was tested 3 h, 1 d, or 3 d after training. Independent groups of bees were used for each retention period. Data for the 3-d retention tests are the same as in previous figures. CS-specific memory is defined as the percentage of bees which responded to the CS but not to the NOd during the retention test. Four of the six drugs (L-DIL, W-7, KN-62, and DDA) induced a highly significant decrease of CS-specific memory with respect to the saline control only during the 3-d retention test. SQ 22536 and MDL 12330A also induced a decrease of CS-specific memory in the 3-d retention test but the level of CS-specific memory reached did not differ significantly from that of the saline group. No differences between the drug-injected group and the saline control were found in the 3-h or the 1-d retention test. The number of animals tested in the different groups is shown above each data point. (***) $P < 0.001$.

two saline controls were run in parallel, one for L-DIL and DDA, and the other for W-7 and KN-62. For the bees injected 10 min after conditioning, each drug-injected group had its own saline control.

In groups injected with W-7, KN-62, or DDA 90 min before conditioning (Fig. 8A), CS-specific memory was similar to that of their corresponding saline control ($q_{\infty,3} < 3.31$, NS for all three drug vs. control comparisons). In contrast, L-DIL induced a significant decrease of CS-specific l-LTM when injected 90 min before conditioning ($q_{\infty,3} = 4.72$, $P < 0.01$). This result indicates

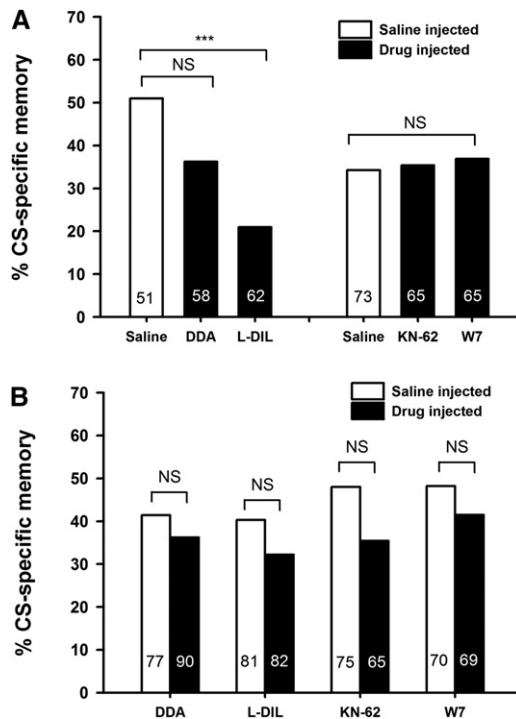


Figure 8. Effect of drug-injection timing on 3-d retention (CS-specific memory). Ninety minutes before conditioning (A) or 10 minutes after conditioning (B), bees were injected with saline solution (white bars) or inhibitors (black bars) (1 mM L-DIL, 1 mM W-7, 2 mM KN-62, or 5 mM DDA). For the bees injected 90 min before conditioning, two saline controls were run in parallel, one for L-DIL and DDA, and the other for W-7 and KN-62. For the bees injected 10 min after conditioning, each drug-injected group had its own saline control. Bees were tested 3 d after training. The sample sizes of each group are indicated within bars. (***) $P < 0.001$, (NS) nonsignificant.

that the effective time window of L-DIL action is longer than those of the other drugs. When injected 10 min after conditioning, none of the drugs affected CS-specific l-LTM so that responses of the drug-injected bees and the saline-injected bees were similar ($q_{\infty,3} < 3.31$, NS for all four drug vs. control comparisons) (Fig. 8B).

Altogether, these results and those shown in Figure 7 indicate that the drugs injected to the bees were effective for blocking l-LTM formation only during a narrow time window, starting with the conditioning (when the odor–sucrose association is first established) and finishing shortly thereafter (as injections 10 min after conditioning are ineffective). The drugs therefore affect the formation but not the retrieval of l-LTM (which took place in our experiments 72 h after conditioning).

Discussion

Our study focused on olfactory learning and memory in the honeybee and aimed at dissecting the contribution of various molecular actors to long-term memory formation. Using the olfactory conditioning of the proboscis extension reflex, we analyzed the performance of 6991 bees injected with different pharmacological inhibitors or saline solution used as a control. We show that CNG channels, CaM, CaMKII, and AC are involved in the formation of a 3-d specific olfactory memory (l-LTM), but are not required for acquisition, STM, MTM (minutes to hours range), or e-LTM (1 d). Our results show that the induction of these pathways is re-

quired during or immediately after conditioning. This is the first comprehensive account demonstrating the involvement of all these molecular actors in the same conditioning protocol (appetitive conditioning of the PER) within the same insect species (the honeybee).

Molecular actors and brain regions in the honeybee

In the honeybee, several brain regions are involved in olfactory learning and memory and exhibit experience-dependent functional and/or structural plasticity (for reviews, see Menzel 1999, 2001; Giurfa 2007; Giurfa and Sandoz 2012). The antennal lobes (ALs), primary olfactory centers receiving olfactory information from olfactory receptor neurons on the antennae, and the mushroom bodies (MBs), multimodal structures receiving processed olfactory information from the ALs as well as visual, mechanosensory, and gustatory input, are the main candidates for hosting long-term memory traces. Both regions exhibit structural modifications after formation of an olfactory l-LTM (Hourcade et al. 2009, 2010; Arenas et al. 2012).

Consistently, several studies point toward the ALs and the MBs as candidate substrates for molecular events leading to l-LTM in the bee brain. For instance, NOS is particularly abundant in the ALs and in the MB calyces (Müller 1996) and the NOS gene is strongly expressed in the Kenyon cells, the constitutive MB neurons (Watanabe et al. 2007). NOS activity is detected in the optic lobes, the mushroom bodies, the central body complex, the lateral protocerebral lobes, and the antennal lobes (Watanabe et al. 2007), and PKA activity is increased in a NO-dependent manner in the ALs following multiple-trial olfactory learning (Müller 2000). Higher levels of immunoreactivity for type-II PKA (PKAII) can be found in the MBs compared to other neuropiles (Müller 1997). Accordingly, the gene of a PKA catalytic subunit is predominantly expressed in the MBs (Eisenhardt et al. 2001). The gene of a membrane-bound AC (*Amac3*) is expressed throughout the brain, but more prominently in the Kenyon cells (Wachten et al. 2006). In *Drosophila melanogaster*, the AC encoded by the *rut* gene, which is critical for olfactory learning, is strongly expressed in MB neurons (Han et al. 1992; Crittenden et al. 1998; Zars et al. 2000; Mao et al. 2004; McGuire et al. 2004). Also in *Drosophila*, a CNG channel-like protein is expressed in AL glomeruli and in the MBs (Miyazu et al. 2000).

The enzymatic activity of CaMKII is enriched in the MBs and central body of the bee compared to the rest of the brain (Kamikouchi et al. 2000). In adults, the phosphorylated form of the enzyme, pCaMKII, is concentrated in a specific subpopulation of Kenyon cells, the noncompact cells (Pasch et al. 2011). Within the olfactory (lip) and visual (collar) subregions of the MB calyx, pCaMKII is colocalized with f-actin in the postsynaptic compartments of the microglomeruli, interaction sites between neurons projecting from primary processing centers (e.g., projection neurons from the ALs) and Kenyon cells. This indicates that pCaMKII is enriched in Kenyon cell dendritic spines, consistent with its potential role in dendritic plasticity. All the molecular actors studied in our work are thus expressed in regions of the bee brain, which have been extensively related with experience-dependent plasticity and associative memory traces.

The role of cAMP–PKA and NO–cGMP signaling pathways in l-LTM formation in honeybees

Both cAMP–PKA and NO–cGMP signaling pathways play an essential role in LTM formation (Eisenhardt 2006). Previous studies on the role of the NO–cGMP pathway in long-term neural plasticity in the mouse hippocampus (Lu et al. 1999; Lu and Hawkins

trial olfactory conditioning induces a significant decrease of e-LTM measured 24 h after conditioning (Müller 2000 [see Fig. 1A therein]). If the PKA antagonist used blocked both types of PKA, then both e-LTM and l-LTM formation would be affected. Note, however, that the decrease in e-LTM found by Müller (2000) upon PKA blockade did not reflect a CS-specific memory as no response to a novel odorant was measured during the tests.

The role of CNG channels in l-LTM formation in honeybees

The connection between the NO-cGMP and the cAMP-PKA signaling pathways remains unclear. Studies in honeybees (Müller 2000) and crickets (Matsumoto et al. 2006) suggested a sequential organization of these pathways. In crickets, CNG channels and $\text{Ca}^{2+}/\text{CaM}$ seem to intervene between the NO-cGMP and the cAMP-PKA pathways during LTM formation (Matsumoto et al. 2006). Consistently, no indication of PKG involvement in LTM formation was found in honeybees (Müller 2000) or crickets (Matsumoto et al. 2006). We therefore propose that in the honeybee, CNG channels and $\text{Ca}^{2+}/\text{CaM}$ act as intermediate actors between the NO-cGMP and the cAMP-PKA pathways to induce l-LTM (Fig. 9).

Specifically, activation of NO-cGMP signaling after multiple-trial conditioning would lead to a binding of cGMP to the CNG channels in the cell membrane, opening them, and making the cell highly permeable to Ca^{2+} . Calcium would then flow into the cell causing a depolarization and ultimately triggering an action potential. Additionally, neuronal excitation could result in activation of an adenylyl cyclase (AC), which would determine an intracellular increase in cAMP; cAMP could also bind to the CNG channels thereby leading to further Ca^{2+} influx, depolarization, and action potentials.

The Ca^{2+} entering through CNG channels would bind to CaM and lead to two possible, nonexclusive processes. First, $\text{Ca}^{2+}/\text{CaM}$ may act on AC, which is sensitive to $\text{Ca}^{2+}/\text{CaM}$ and G-protein stimulation (Livingstone et al. 1984; Levin et al. 1992). Stimulation of AC would induce an increase in cAMP levels, thereby providing the basis for prolonged PKA activation underlying l-LTM formation (Müller 2000). Second, $\text{Ca}^{2+}/\text{CaM}$ may directly act on CaMKII, which has also been related to LTM formation (Akalal et al. 2010). The role of these two molecular actors, AC and CaMKII, is discussed in the next sections.

The role of AC in l-LTM formation in honeybees

Despite its suggested role as coincidence detector in associative learning (Gervasi et al. 2010), our results showed that AC was dispensable for acquisition, STM, MTM, and e-LTM formation (see Figs. 6 and 7). Clearly, both learning and STM, reflected by the response in consecutive conditioning trials spaced by 10 min, were unaffected by AC blockade. Similarly, retention 3 h and 1 d after conditioning were also unaffected. This result was unexpected because if AC is a molecular coincidence detector integrating CS and US inputs and thus facilitating associative learning and memory, its inhibition should suppress not only all memory phases but also olfactory learning itself. A possible explanation for our findings is that the lack of suppressive effects of the three AC antagonists used—DDA, SQ 22536, and MDL 12330A—was due to the existence of several ACs with different antagonist affinities and playing different roles for different memory phases. For instance, from the three antagonists used, DDA yielded the clearest effect in the 72-h retention test: all three drugs induced a decrease in CS-specific memory but only the one induced by DDA reached significance.

In *Drosophila melanogaster*, a total of 10 genes encoding ACs have been identified from which the $\text{Ca}^{2+}/\text{CaM}$ -regulated rutabaga (*rut*) is the best characterized AC enzyme (Livingstone et al. 1984; Levin et al. 1992). In the honeybee, a first gene encoding an AC protein, AmAC3, was identified (Wachten et al. 2006) and its biochemical and pharmacological properties were characterized in vitro (Fuss et al. 2010). Moreover, recent studies indicate the presence of other ACs besides AmAC3 (Balfanz et al. 2012). Bioinformatics and functional expression allowed two additional genes encoding membrane-bound (tm)ACs, AmAC2t and AmAC8, to be characterized. Unlike the common structure of tmACs, AmAC2t lacks the first transmembrane domain. Three additional tmACs and one soluble AC-encoding gene were also identified using bioinformatics (Balfanz et al. 2012). These results indicate that the AC-gene family of the honeybee is comparably as large as that in other species (e.g., *Drosophila*; see above). Thus, the three inhibitors used may not be equally effective for all existing ACs, targeting only those ACs that intervene in the formation of l-LTM (termed “AC_{l-LTM}” in Fig. 9) but not those that participate in the formation of earlier memory phases (termed “AC_{e-LTM}” in Fig. 9). The former (“AC_{l-LTM}”) could be activated, for instance, through multiple spaced conditioning trials, resulting in an increase of PKA activation, which would target the transcription factor CREB (AmCREB) within the cell nucleus (Fig. 9; Eisenhardt et al. 2003). The latter (“AC_{e-LTM}”), activated, for instance, through a single conditioning trial, could induce PKA activation, targeting translation at the ribosomal level via an unknown pathway, but not transcription (Fig. 9). Our results thus suggest heterogeneity in the role of different ACs in memory formation and ask for a precise characterization of the different ACs identified so far in the honeybee.

The role of CaMKII in l-LTM formation in honeybees

$\text{Ca}^{2+}/\text{CaM}$ may directly act on CaMKII, which has been repeatedly related to LTM formation. In the pond snail *Lymnaea stagnalis*, for instance, CaMKII is necessary for acquisition and late consolidation, but not early or intermediate consolidation or retrieval of LTM (Wan et al. 2010). In *Drosophila*, CaMKII is necessary for courtship-associated learning and memory (Mehren and Griffith 2004) and for LTM formation following odor-shock pairing (Akalal et al. 2010). In the latter case, flies that experienced spaced odor-shock pairings exhibit an increase in calcium influx into a subclass of Kenyon cells, the γ neurons, when tested between 18 and 48 h after training. This increase relates to the formation of LTM, which occurs between 9- and 18-h post-training and persists until 48-h post-training, and is blocked by a dCREB repressor molecule or a CaMKII hairpin RNA (Akalal et al. 2010). Thus, in snails, flies, and bees, protein-dependent forms of LTM require the normal activity of CaMKII.

Molecular cascades and memory phases in honeybees

In honeybees, several memory phases have been characterized following associative, appetitive olfactory learning (Menzel 1999; Eisenhardt 2006). Both single- and multiple-trial conditioning induce STM in the seconds-to-minutes range, and MTM, in the minutes-to-hours range. Memory induced by a single-trial conditioning decays over time, is sensitive to amnesic treatments (Menzel et al. 1974; Erber et al. 1980), and is independent of translation and transcription. Three-trial conditioning, as performed in our work, induces a stable, long-lasting memory that can be divided into e-LTM, which depends on translation, and l-LTM, which is insensitive to amnesic treatments and requires both translation and transcription (Menzel 1999). Both LTM types are impaired by blocking PKA or the NO-cGMP pathway during conditioning (Müller 2000), but two parallel molecular pathways,

one leading to e-LTM and the other to l-LTM, seem to be triggered by independent PKA-induced processes during conditioning (Friedrich et al. 2004). The present study provides a further differentiation between these two forms of LTM, namely that l-LTM formation requires CNG channels, calmodulin, AC, and CaMKII, while e-LTM formation does not require, in principle, any of these molecules, although caution is required in the case of AC, as different ACs may intervene in the formation of different memory phases (see Fig. 9). This dissociation may be based on the existence of different subclasses of Kenyon cells expressing or not the key molecular actors necessary for l-LTM formation. For instance, the pCaMKII protein is predominantly concentrated in noncompact Kenyon cells (Pasch et al. 2011). The careful mapping of the studied molecules in the bee brain may allow finding the neurons responsible for e-LTM and l-LTM formation.

Alternatively, all molecules necessary for the formation of different memory phases may be present in all Kenyon cells so that conditioning parameters, such as the stimulation schedule of CS–US pairings, would be critical for the activation of different signaling pathways. For instance, a single conditioning trial (i.e., a single CS–US pairing) would determine the activation of signaling pathways leading to translation and e-LTM (as well as earlier memory phases such as STM and MTM) while multiple spaced conditioning trials could activate, through repetitive CS–US pairings, signaling pathways leading to transcription and l-LTM (Fig. 9).

Addressing CS-specific memories in studies on memory formation

Most previous experiments on memory retention in bees used absolute conditioning in which a single odor is paired with sucrose reward. Usually, retention tests consisted of one or various presentations of the odor CS, without presenting any novel odorant. This procedure is questionable as the bee's response to the CS may also include some non-CS-specific components (Matsumoto et al. 2012). We therefore want to underline the importance of presenting a novel odor to animals trained in absolute-conditioning protocols, so that the specific contribution of associative memories can be studied. In our experiments, the level of nonspecific responses remained unchanged after most treatments. In only one case did drug-injected bees display a significant increase in their nonspecific response level (injection of CaM blocker R24571) (Fig. 4F). Otherwise, all drugs directly affected the CS-specific component of l-LTM, indicating that they specifically blocked the formation of l-LTM for the CS–US association. Previous studies on bees reported only partial blockade of associative olfactory memory after pharmacological interference with NOS, sGC, PKA, proteases, translation, or transcription (Müller 1996, 2000; Grünbaum and Müller 1998; Wüstenberg et al. 1998). Yet these studies assessed only CS responses during retention so that the drug-resistant LTM component observed could be, in part, nonspecific.

Conclusion

This work demonstrates the necessity of the NO–cGMP pathway, and of specific molecules, such as cyclic nucleotide-gated channels (CNG), calmodulin (CaM), adenylyl cyclase (AC), and Ca²⁺/calmodulin-dependent protein kinase (CaMKII) for the formation of transcription-dependent l-LTM. The emerging model suggests that different pathways, involving different molecular actors, lead to translational and transcriptional processes, and thus to e-LTM and/or l-LTM, after CS–US coincidence detection. Figure 9 illustrates these pathways in the case of a Kenyon cell, an intrinsic neuron of the MBs. Two possibilities are shown

for US signaling (“US pathway”) through the octopaminergic VUMmx1 neuron, which mediates the reinforcing properties of sucrose solution in the bee brain (Hammer 1993). In one case, octopamine released by VUMmx1 binds to the octopamine G_q-coupled receptor AmOA1 resulting in an intracellular increase of Ca²⁺ from internal Ca²⁺ stores and in a subsequent activation of an AC (AC_{e-LTM}, see above) involved in the formation of e-LTM; in the other case, octopamine binds to an unknown octopamine receptor (OAR) that is coupled to a G_s-protein activating AC_{e-LTM}.

Presynaptic CS signaling to the Kenyon cell occurs via a projection neuron which releases the excitatory neurotransmitter acetylcholine, binding, for instance, to a nicotinic receptor (NACHR) of the Kenyon cell. This binding determines a Ca²⁺ influx and a subsequent activation of the AC_{e-LTM}, which would thus act as a CS–US coincidence detector upon double activation via CS and US signals (Fig. 9). AC activation determines conversion from ATP to cAMP, which in turn activates the PKA specific to the e-LTM pathway (PKA_{e-LTM}). As suggested above, this PKA might directly regulate translational activity at the ribosome level, supporting e-LTM.

Excitation of the Kenyon cell may lead to release of an excitatory neurotransmitter (acetylcholine or glutamate) to a postsynaptic cell (Grünwald 1999) leading to a retrograde NO neurotransmission back to the Kenyon cell (Müller 1996). This pathway would lead to transcription-dependent l-LTM and would involve the molecular actors studied in our experiments. Thus, Ca²⁺ influx resulting from acetylcholine or glutamate excitatory neurotransmission would activate CaM, which in turn activates a NO synthase (NOS). NO is synthesized by NOS and diffuses into the Kenyon cell where it activates a soluble guanylyl cyclase (sGC); sGC converts GTP to cGMP, which activates CNG channels, thereby favoring Ca²⁺ influx, depolarization, and CaM activation. CaM could activate CaMKII on the one hand, and a form of AC specific to the l-LTM pathway (AC_{l-LTM}) on the other hand. The latter would determine an increase in cAMP and a prolonged activation of a PKA form specifically of this l-LTM pathway (PKA_{l-LTM}). Both CaM-activated pathways would target within the nucleus the transcription factor CREB (AmCREB), thus resulting in *de novo* protein synthesis required for l-LTM.

This model needs to be tested through a series of experiments that are necessary to confirm or refute the steps proposed in Figure 9. For instance, given that NOS activation precedes CNG activation, with GTP conversion to cGMP via sGC as an intermediate step, blocking NOS and CNG channels should yield the same result as blocking just NOS or CNG channels. Also, inhibition of NOS via L-NAME could theoretically be rescued by cGMP as a CNG channel agonist, or by other substituted cGMP analogs which act as efficient CNG channel agonists (Wei et al. 1998).

Furthermore, as CNG channel activation is upstream of CaM, CaMKII, and AC_{l-LTM}/PKA, injection of the CNG channel antagonist L-DIL should remove l-LTM per se so that further injection of CaM, CaMKII, or AC_{l-LTM}/PKA antagonists should not induce further decreases of l-LTM. Also, because CaMKII and AC_{l-LTM}/PKA constitute parallel pathways triggered by CaM, injection of the AC_{l-LTM} antagonist DDA and of the CaMKII antagonist KN-62 should induce a more drastic, additive reduction of l-LTM.

These experiments are only some examples from a vast possible agenda necessary to unravel the molecular underpinnings of memory formation in honeybees. Some of the features of our model may be wrong in their particulars while some other features may be relevant only under certain experimental conditions pertaining to conditioning schedules and parameters (e.g., CS/US duration and overlap, interstimulus interval, trial number, etc.). Future studies based on the present work will refine and clarify these issues.

Materials and Methods

Animals

Female honeybee workers *Apis mellifera* caught upon departure from the hive were cooled on ice for 5 min until they ceased movements. They were then individually harnessed in small metal tubes so that only the head protruded. Mouthparts and antennae could freely move. After being fed with 4 μ L of a 50% (weight/weight) sucrose solution, bees were left undisturbed for ~4 h. Fifteen minutes before olfactory conditioning, each subject was checked for intact proboscis extension reflex (PER) by lightly touching the antennae with a toothpick imbibed with 50% sucrose solution without subsequent feeding. Hungry, motivated bees respond to this stimulation by extending reflexively the proboscis (PER) to reach out to and lick the sucrose. Extension of the proboscis beyond the virtual line between the open mandibles was counted as a response. Animals that did not exhibit a PER at this stage were not used in the experiments (<5%).

Drugs

Depending on their solubility in water, drugs were dissolved in either 1 μ L of honeybee saline solution (130 mM NaCl, 6 mM KCl, 4 mM MgCl₂, 5 mM CaCl₂, 160 mM sucrose, 25 mM glucose, 10 mM HEPES; henceforth "saline") or 1 μ L of saline containing 0.05%–0.5% dimethyl sulfoxide (DMSO). Accordingly, control bees were injected with either 1 μ L of saline or 1 μ L of saline containing 0.05%–0.5% DMSO. Injections were performed into the hemolymph of the thorax using a 10- μ L microsyringe (WPI).

The drugs used were: L-NAME, an inhibitor of NOS, the NO synthase (N ω -nitro-L-arginine methyl ester); ODQ, a soluble guanylyl cyclase (sGC) inhibitor (1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one); CHX, an inhibitor of protein synthesis (cycloheximide); L-DIL, a CNG channel-inhibitor (L-*cis*-diltiazem hydrochloride); W-7, TFP, and R24571, three CaM antagonists (N-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide hydrochloride, trifluoperazine, and calmidazolium, respectively); KN-62, a CaMKII inhibitor (1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine); and three AC inhibitors, DDA, SQ 22536, and MDL 12330A (respectively, 2',5'-dideoxyadenosine, 9-[tetrahydrofuryl]-adenine, and *cis*-N-[2-phenylcyclopentyl]-azacyclotridec-1-en-2-amine) (see Table 1).

The doses of L-NAME and ODQ (1.3 mg/kg and 0.9 mg/kg body weight, respectively) were chosen according to previous works in which these drugs acted as efficient NOS and sGC inhibitors in the honeybee nervous system (Müller 1996, 2000). The dose of CHX (140.7 mg/kg body weight) was three times larger than that reported as being ineffective for blocking memory in bees (42 mg/kg body weight) (see Wittstock et al. 1993).

Chemicals were purchased from Sigma-Aldrich, except L-DIL (from Biomol), SQ 22536 and MDL 12330A (both from Merck-Millipore). ODQ, W-7, R24571, and KN-62 were dissolved in saline containing 0.05%–0.5% DMSO. The other drugs were dissolved in saline alone. Injections were performed 20 or 90 min before, or 10 min after conditioning. Experimental groups that were run in parallel and that had different drug concentrations, and thus different amounts of DMSO, had a corresponding saline-control group which contained the highest amount of DMSO.

Stimulation setup

For olfactory conditioning, odorants were delivered using an olfactometer sending a constant clean airstream in which odor pulses of known duration could be introduced. Each odorant was applied onto a filter paper placed within a syringe connected to the odor-delivery setup. The airstream was produced by an air pump (Rena Air 400) and directed to the relevant syringes by means of electronic valves (Lee Company S.A.) controlled by a computer. In the absence of odorant stimulation, the airstream passed through a syringe containing a clean piece of filter paper (clean airstream). During olfactory stimulation, the airstream was directed to a syringe containing a filter paper loaded with odorant. After 4-sec stimulation, the airstream was again redirect-

ed to the odorless syringe until the next olfactory stimulation. The whole setup was placed in front of an air extractor, which impeded the accumulation of residual odors after delivery of an olfactory stimulus.

Stimuli

Two odorants, 1-nonanol and 2-hexanol, were used as conditioned stimuli (CS) in a balanced manner, i.e., for half of the bees 1-nonanol was the CS, while for the other half 2-hexanol was the CS. These two odorants are well learned by bees and induce low cross-generalization responses in appetitive olfactory conditioning (Guerrieri et al. 2005). Bees trained with either CS were afterward tested with both odorants—the CS and the novel odor (NOd)—to assess the specificity of the olfactory memories evoked in the retention tests. Fifty percent sugar solution (weight/weight) was used throughout as the unconditioned stimulus (US), and was delivered to the antennae and the proboscis by means of a toothpick.

Conditioning procedure

Bees injected with different drugs or with saline solution (control bees) were subjected to olfactory conditioning of the proboscis extension response (PER) (Takeda 1961; Bitterman et al. 1983; Giurfa and Sandoz 2012). In this conditioning protocol, the presentation of an odorant immediately before sucrose solution (forward pairing) results in an association between odorant and sucrose that triggers PER in subsequent, nonrewarded retention tests. This effect is clearly associative and involves classical conditioning (Bitterman et al. 1983).

Bees were conditioned during three CS–US trials separated by an inter-trial interval of 10 min. This protocol has been shown to induce the formation of robust LTM in bees (Menzel 1999). Each conditioning trial lasted 30 sec. A trial started when a harnessed bee was placed between the olfactometer and an air extractor for 13 sec to allow familiarization with the training situation. Thereafter, the CS was released for 4 sec. Three seconds after CS onset, the antennae were stimulated with sucrose solution (US), leading to PER. The bee was allowed to feed for 3 sec. Therefore, stimulus overlap was 1 sec and interstimulus interval 3 sec. The bee was then left in the conditioning place for 11 sec and then removed.

During each trial, we recorded whether the bee extended its proboscis after CS onset and before US onset (conditioned response). Bees that responded less than twice to the US (out of three presentations) during acquisition, thus exhibiting imperfect unconditioned responses, were excluded from the analyses. They represented 7.7% of saline-injected bees ($n = 2293$) and 8.9% of drug-injected bees ($n = 4698$). No differences were ever found in rates of unconditioned responses between drug-treated animals and controls.

Retention tests

Bees were presented with the CS and with the NOd in order to assess the specificity of the retrieved olfactory memories (Matsumoto et al. 2012). Retention tests were separated by 10 min. Half of the bees first received the CS followed by the NOd while the other half experienced the reversed sequence. Odorant stimulation was identical to that of conditioning trials (4 sec) but no US was given. Retention tests were performed 3 h (MTM), 1 d (e-LTM), or 3 d (l-LTM) after the last conditioning trial. Independent groups of bees were tested at these different periods. After the two retention tests, PER to the US was checked once again. Animals unable to show PER at this point were not considered for the analyses because their lack of response to the odors cannot be necessarily ascribed to a memory deficit but to fatigue.

Bees tested for retention 1 d or 3 d after conditioning were subjected to specific handling procedures to ensure survival. Bees tested 1 d after conditioning were fed until satiation with 50% sucrose solution and kept in the harnessing tubes until the

test. Feeding started at least 60 min after the end of training. Bees tested 3 d after conditioning were not kept in their tubes until the retention tests as this procedure induces high mortality. They were first painted on the thorax with watercolors allowing individual identification and were then placed in groups of ~30 individuals in small wooden cages (65 × 70 × 25 mm) containing a diet of food (mixture of 50% sucrose and 50% honey) as well as water ad libitum. Cages were kept in a dark and humid box at 33°C for ~72 h. On the morning of the third day after conditioning, bees were cooled on ice and reharnessed individually in the metal tubes. Retrieval tests were performed after 5-h food deprivation.

Statistical analysis

We checked that the treatment neither affected survival nor PER integrity. Only bees that survived the entire experiment and showed a PER to sucrose solution at the end of retention tests (see above) were included in the analyses. In the groups that were subjected to the 3-d retention test, 21.8% of the bees died between the end of conditioning and the beginning of the tests (25.4% in control groups, 20.3% in drug-injected groups). From the remaining bees, 4.8% did not show a PER to the US at the end of the retention tests (6.4% in control groups, 4.2% in drug-injected groups). In the groups subjected to the 1-d retention test, 18.5% of the bees died between the end of conditioning and the beginning of the tests (20.7% in control groups, 17.1% in drug-injected groups). From the remaining bees, 6.8% did not show a PER to the US at the end of the retention tests (3.8% in control groups, 8.6% in drug-injected groups).

ANOVA for repeated measurements was used to analyze acquisition performances within or between groups. ANOVA procedures are allowed in the case of binary responses such as PER if comparisons imply equal cell frequencies and at least 40 degrees of freedom of the error term (Lunney 1970). To compare response levels to the CS and the novel odorant in the tests, a McNemar test was used within each group. Multiple comparisons between CS-specific memory levels were performed by means of a Tukey test adapted for proportions, on which a Freeman and Tukey angular transformation was applied (Zar 1999, pp. 563–565). All statistical analyses were carried out using Statistica 5.5 (StatSoft), and differences were considered significant if the *P*-value was <0.05.

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