Redefining Single-Trial Memories in the Honeybee

Graphical Abstract

Highlights

- A single olfactory conditioning trial induces long-term memories in honeybees
- These memories differ in their dependency on translation and transcription
- 4 h after conditioning, the memory depends only on translation processes
- One and three days later, the memory depends both on translation and transcription

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In Brief

Villar et al. show that a single olfactory conditioning trial in bees induces not only transient memories, but also protein-synthesis-dependent long-term memories that appear earlier than expected. 4 h after conditioning, the olfactory memory depends on translation. One and three days later, it depends both on transcription and translation.
Redefining Single-Trial Memories in the Honeybee

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SUMMARY

Research on honeybee memory has led to a widely accepted model in which a single pairing of an odor stimulus with sucrose induces memories that are independent of protein synthesis but is unable to form protein-synthesis-dependent long-term memory (LTM). The latter is said to arise only after three or more pairings of odor and sucrose. Here, we show that this model underestimates the capacity of the bee brain to form LMTs after a unique appetitive experience. Using state-of-the art conditioning setups and individual-based analyses of conditioned responses, we found that protein-synthesis-dependent memories are formed already 4 h after the single conditioning trial and persist even 3 days later. These memories (4 h, 24 h, and 72 h) exhibit different dependencies on transcription and translation processes. Our results thus modify the traditional view of one-trial memories in an insect with a model status for memory research.

INTRODUCTION

Learned information is typically encoded and stored in the nervous system, from where it can be retrieved to respond appropriately to events previously experienced. Memory, the sum of these processes, can be classified according to multiple criteria, one of which is durability (Milner et al., 1998; Kandel, 2001; Squire, 2009). Short-lasting and long-lasting memories are distinguished in most living animals, a classification that is sustained by the different biological processes underlying these memory forms (Goelet et al., 1986; Kandel, 2001). Accordingly, long-term memory (LTM) is typically defined as a durable and robust memory that is stabilized in time based on a consolidation phase requiring protein synthesis (Squire and Davis, 1981; Davis and Squire, 1984). On the contrary, short-term memory (STM) decays rapidly over time and does not require protein synthesis. The mechanisms mediating these two types of memory are independent and may occur in parallel (Izquierdo et al., 1998; Isabel et al., 2004; Trannoy et al., 2011).

Invertebrates have made fundamental contributions to the study of memory (Carew and Sahley, 1986; Menzel, 1999; Heisenberg, 2003; Giurfa and Sandoz, 2012; Giurfa, 2013). Among them, the honeybee emerged as a standard model for the distinction between memory phases due to its remarkable learning and memory capacities and the parallels existing between the temporal organization of its memory and that of vertebrates (Menzel and Müller, 1996; Menzel, 1999; Müller, 2012; Eisenhardt, 2014). Olfactory STM and LTM have been profusely documented in honeybees using a learning protocol termed the olfactory conditioning of the proboscis extension response (PER) (Takeda, 1961; Bitterman et al., 1983; Giurfa and Sandoz, 2012; Matsumoto et al., 2012). In such a protocol, hampered bees learn to associate an odorant (the conditioned stimulus [CS]) with a reward of sucrose solution (the unconditioned stimulus [US]). After successful learning, bees exhibit the appetitive PER to the odorant that anticipates the food.

Extensive research on olfactory memory in bees led to an established model of the memories existing in this insect (Menzel, 1999, 2012; Eisenhardt, 2006; Müller, 2012). This model posits that a single learning trial (i.e., a single pairing of an odor with sucrose reward) leads to a STM (in the range of seconds to minutes) and a mid-term memory (MTM; in the range of minutes to hours). Interestingly, MTM was not addressed specifically after a single conditioning trial, and statements about it refer mostly to findings obtained after multiple conditioning trials. Yet, it is commonly accepted that both STM and MTM are available after one conditioning trial and that both memory phases are susceptible to various interference treatments, such as local cooling and extinction, but are insensitive to inhibition of protein synthesis (Menzel, 1999). Subsequently, memory decays considerably over time, and even if it can sometimes be evoked, it remains insensitive to protein-synthesis inhibition (Grünbaum and Müller, 1998; Friedrich et al., 2004; Pamir et al., 2014). On the contrary, multiple learning trials have been shown to induce not only STM and MTM, but also a 24-h memory termed early LTM (e-LTM) and a late LTM (l-LTM) that can be retrieved several days after training (e.g., 72 h post-conditioning). While e-LTM depends on translation processes, l-LTM depends on both
transcription and translation processes (Wüstenberg et al., 1998; Menzel, 1999, 2012). Thus, repeated trials are considered necessary to trigger the molecular cascades enabling L-LTM consolidation via protein synthesis (Menzel, 1999, 2001, 2012). Here, we focused on the memories induced by a single conditioning trial, which has the advantage of allowing a clear separation between consolidation and retrieval (Izquierdo and Medina, 1997; Izquierdo et al., 2002). We coupled one-trial olfactory conditioning of PER with injections of emetine (Eme) (translation inhibitor) or actinomycin D (transcription inhibitor) into the bee brain via the ocellar tract (Tedjakumala et al., 2014). We used puromycin labeling to assess the effect of Eme on protein synthesis at different periods following injection. This method, which is based on the incorporation of puromycin into nascent peptide chains, has been used to detect changes in protein synthesis levels in various animal models, including mammals, Drosophila, and the honeybee (Schmidt et al., 2009; Marter et al., 2014; Deliu et al., 2017).

Injections of Eme or PBS (control solution) via the ocellar tract were performed 5 min, 30 min, or 60 min before injection of puromycin (1 μl, 1000 μg/ml) (Figure 1A). 45 min after puromycin injection, brains were removed and puromycin labeling was quantified by western blots of an equal number of proteins from individual bee brains. The intensity of puromycin labeling following PBS injection was used as a positive control (100% signal), while the labeling obtained in the absence of puromycin with Eme or PBS was used as a negative control. In every case, the labeling was normalized to its correspondent tubulin labeling and then normalized again to its positive control located on the same blot.

Puromycin incorporation decreased at least 60% when Eme was injected 5, 30, and 60 min before puromycin when compared to the corresponding PBS controls (Figures 1B and 1C). Comparing groups injected with Eme or PBS showed that Eme injection significantly decreased puromycin incorporation in all groups (Student’s t test; 5 min: t = 7.62, degrees of freedom [df] = 10, p < 0.001; 30 min: t = 3.34, df = 6, p < 0.05; 60 min: t = 8.37, df = 6, p < 0.001). Thus, Eme inhibited protein synthesis in the bee brain for at least 1.75 h after injection (60 min between Eme and puromycin injections + 45 min before tissue extraction).

Memories Retrieved 4 h, 24 h, and 72 h after Single Conditioning Trial Depend on Protein Synthesis
We used a single conditioning trial to train bees to associate an odorant (CS) with a reward of sucrose solution (US) (Figure 2A). The translation inhibitor (Eme) or its vehicle (Veh) PBS was injected 30 min before the conditioning trial. Memory retention was measured 1 h, 4 h, 24 h, or 72 h after training by presenting the CS and a novel odorant (NOd), which allowed determining the specificity of the CS memory (Matsumoto et al., 2012). Figure 2A). Independent groups of bees were used for each retention test. Based on previous results, we expected that the single conditioning trial would induce memory at shorter delays post-conditioning and, eventually, a decaying, residual memory retrievable at longer delays, which should be insensitive to a blockade of protein synthesis (Grünbaum and Müller, 1998; Friedrich et al., 2004; Pamir et al., 2014). Thus, bees should respond strongly to the CS at 1 h and 4 h (which, in the current model of bee memory, correspond to MTM) but not (or only weakly) at 24 h (e-LTM) or at 72 h (l-LTM).

Figure 2B shows that Veh-injected bees exhibited robust memory retention at 1 h, 4 h, 24 h, and 72 h after the single conditioning trial. In all cases, responses to the CS were significantly higher than were generalization responses to the NOd (McNemar’s tests; Veh 1 h: χ² = 22, df = 1, p < 0.001; Veh 4 h: χ² = 27, df = 1, p < 0.001; Veh 24 h: χ² = 50, df = 1, p < 0.001; Veh 72 h: χ² = 9, df = 1, p < 0.01). These results show that it is possible to induce memory formation and retention up to
In the current understanding of honeybee memory, the 1-h and 4-h memories are described as MTMs (Menzel, 2012), a memory form that was characterized using multiple-trial conditioning and that is independent of protein synthesis (Grünbaum and Müller, 1998; Müller, 2013). On the contrary, the 24-h and 72-h memories are described as LTM that was characterized using multiple-trial conditioning and is dependent on protein synthesis (Friedrich et al., 2004). Following this view, the 1-h memories are described as LTMs and are said to both depend on and be impaired by Eme injection. Following this view, the 1-h memories are described as LTMs and are said to both depend on and be impaired by Eme injection.

Figure 2 shows that significant retention was found at 1 h, 4 h, and 24 h, but not at 72 h after the single conditioning trial. This is consistent with the idea that bees tested 1 h after conditioning are in the same conditions during training and the test. The CS-specific memory of the Eme/Eme group was significantly higher than the responses of the Eme group at 1 h. This demonstrates that Eme impaired memory retention not only at 72 h, but also at 4 h and 24 h after conditioning.

An alternative explanation to the effect of Eme on retention at 4 h and 24 h, but not at 1 h, might be that bees tested 1 h after the single conditioning trial are not in the same conditions as bees tested at longer intervals. If, for instance, Eme changes the perception of the conditioned odor, bees would perceive the conditioned odor in the 1-h test in the same way as during training, thus facilitating a response because Eme is still active. If, for instance, Eme changes the perception of the conditioned odor, bees would perceive the conditioned odor in the 1-h test in the same way as during training, thus facilitating a response because Eme is still active.

3 days after a single conditioning trial and that even at these long retention intervals, the memory expressed is CS specific.

Figure 2B shows that significant retention was found at 1 h, 4 h, and 24 h, but not at 72 h after the single conditioning trial. Eme-injected bees responded more to the CS than to the NOd in the first three tests but not in the latter (McNemar's tests; \( \chi^2 = 24, df = 1, p < 0.001 \); Eme 4 h: \( \chi^2 = 14, df = 1, p < 0.001 \); Eme 24 h: \( \chi^2 = 18.2, df = 1, p < 0.001 \); Eme 72 h: \( \chi^2 = 2, df = 1, p = 0.157 \); independent groups used for each test). Yet, this analysis may hide further effects of Eme, as it focuses on population responses instead of on individual responses (Pamir et al., 2011). To refer the analysis to individual performances in both the Eme and Veh groups, we quantified the percentage of bees exhibiting CS-specific responses (i.e., the bees that correctly responded to the CS and not to the NOd) (Figure 2C). An impact of Eme on retention would be visible through a significant decrease of CS-specific memory in the Eme group, compared to the Veh group. 1 h after conditioning, no differences were found between the Veh and the Eme groups (chi-square test; \( \chi^2 = 0.373, df = 1, p = 0.542 \)), thus showing that the 1-h memory was insensitive to the Eme treatment. Yet, in the other retention tests, responses of the Veh group were significantly higher than the responses of the Eme group (4 h: \( \chi^2 = 5.81, df = 1, p < 0.05 \); 24 h: \( \chi^2 = 30.5, df = 1, p < 0.001 \); 72 h: \( \chi^2 = 4.39, df = 1, p < 0.05 \)). This demonstrates that Eme impaired memory retention not only at 72 h, but also at 4 h and 24 h post-conditioning.

Figure 3 shows that the pre-training injection had a significant effect on performance, while the same injection before the 24-h test had no effect (two-way ANOVA; factor injection pre-training: \( F_{(1, 104)} = 15.1, p < 0.001 \); factor injection pre-test: \( F_{(1, 104)} = 1.73, p = 0.19 \); interaction: \( F_{(1, 104)} = 0.02, p = 0.97 \)). The second injection of Eme 30 min before the 24-h test had no effect (two-way ANOVA; factor injection pre-training: \( F_{(1, 104)} = 24, df = 1, p < 0.001 \); Eme 24 h: \( \chi^2 = 18.2, df = 1, p < 0.001 \); Eme 72 h: \( \chi^2 = 2, df = 1, p = 0.157 \); independent groups used for each test). Yet, this analysis may hide further effects of Eme, as it focuses on population responses instead of on individual responses (Pamir et al., 2011). To refer the analysis to individual performances in both the Eme and Veh groups, we quantified the percentage of bees exhibiting CS-specific responses (i.e., the bees that correctly responded to the CS and not to the NOd) (Figure 2C). An impact of Eme on retention would be visible through a significant decrease of CS-specific memory in the Eme group, compared to the Veh group. 1 h after conditioning, no differences were found between the Veh and the Eme groups (chi-square test; \( \chi^2 = 0.373, df = 1, p = 0.542 \)), thus showing that the 1-h memory was insensitive to the Eme treatment. Yet, in the other retention tests, responses of the Veh group were significantly higher than the responses of the Eme group (4 h: \( \chi^2 = 5.81, df = 1, p < 0.05 \); 24 h: \( \chi^2 = 30.5, df = 1, p < 0.001 \); 72 h: \( \chi^2 = 4.39, df = 1, p < 0.05 \)). This demonstrates that Eme impaired memory retention not only at 72 h, but also at 4 h and 24 h post-conditioning.

An alternative explanation to the effect of Eme on retention at 4 h, 24 h, and 72 h, but not at 1 h, might be that bees tested 1 h after the single conditioning trial are not in the same conditions as bees tested at longer intervals. If, for instance, Eme changes the perception of the conditioned odor, bees would perceive the conditioned odor in the 1-h test in the same way as during training, thus facilitating a response because Eme is still active 1 h after injection (Figure 2B). On the contrary, if Eme has worn off at longer intervals post-conditioning, the conditioned odor could be perceived as being different from that experienced during training, thus inducing a decrease in response. To address this perceptual hypothesis, we performed a control experiment in which bees were subjected to two injections: one 30 min before the single conditioning trial and another 30 min before the 24-h test. In this way, bees were in identical conditions both during training and during the retention test. Four groups were trained and tested in parallel (Veh/Veh, Veh/Eme, Eme/Veh, and Eme/Eme). Figure S1 shows that the pre-training injection had a significant effect on performance, while the same injection before the 24-h test had no effect (two-way ANOVA; factor injection pre-training: \( F_{(1, 104)} = 15.1, p < 0.001 \); factor injection pre-test: \( F_{(1, 104)} = 1.73, p = 0.19 \); interaction: \( F_{(1, 104)} = 0.02, p = 0.97 \)). The second injection of Eme 30 min before the test did not re-establish responding in the Eme/Eme group, despite the fact that animals were in the same conditions during training and the test. The CS-specific memory of the Eme/Eme group remained low and similar to that of the group having received first Eme and then the Veh (Eme/Veh). Thus, the decrease in performance in the 24-h test was not due to a perceptual problem induced by Eme, but rather to the blockade of protein synthesis that affected similarly the two groups that received Eme 30 min before conditioning. The group that received the pre-training injection of Veh and that of Eme 30 min before the 24-h test (Veh/Eme) had intact memory, which was similar to that of the Veh/Veh group. In addition, this shows that the protein synthesis necessary for LTM consolidation was no longer present 23.5 h after the single conditioning trial.
dependent memory exists already at this time. As LTMs are typically defined as protein-synthesis dependent, the 4-h memory can also be classified as a LTM.

Different Translation and Transcription Dependencies of the 4-h, the 24-h, and the 72-h Memories Induced by a Single Conditioning Trial

As the 4-h memory depends on the translation of RNA transcripts, it coincides with the e-LTM originally defined for the honeybee and ascribed to a 24-h period (Menzel and Müller, 1996; Wüstenberg et al., 1998; Müller, 2013; Eisenhardt, 2014). Yet, this memory could also depend on transcription processes and thus exhibit characteristics similar to the I-LTM, originally attributed to periods equal to or longer than 72 h in the honeybee (Menzel and Müller, 1996; Wüstenberg et al., 1998; Müller, 2013; Eisenhardt, 2014). To study the dependency of memory on transcription processes, we injected the transcription inhibitor actinomycin D (ActD) into the bee brain 30 min before a single conditioning trial and measured memory retention 4 h, 24 h, or 72 h later (Figure 3A). We focused on these memories, as they were shown to depend on translation processes in the previous experiment.

Figure 3B shows the population responses to the CS and to the NOd in the retention tests. Again, the Veh group exhibited significant retention not only at 4 h, but also at 24 h and 72 h after conditioning. In all three tests, responses to the CS were significantly higher than to the NOd (Figure 3B; Veh 4 h: $\chi^2 = 21$, df = 1, $p < 0.001$; 24 h: $\chi^2 = 25$, df = 1, $p < 0.001$; 72 h: $\chi^2 = 12$, df = 1, $p < 0.001$). ActD-injected bees exhibited significant retention 4 h and 24 h after the single conditioning trial but not 72 h after it (Figure 3B; ActD 4 h: $\chi^2 = 21$, df = 1, $p < 0.001$; 24 h: $\chi^2 = 10.3$, df = 1, $p < 0.01$; 72 h: $\chi^2 = 2.67$, df = 1, $p = 0.10$).

Although these results seem to circumscribe the necessity of transcription processes to the 72-h memory, the analysis of CS-specific memory again revealed overlooked effects of ActD. Bees in the Veh and the ActD groups did not differ in the 4-h retention test (Figure 3C; $\chi^2 = 0.0778$, df = 1, $p = 0.780$), thus confirming that the 4-h memory was translation but not transcription dependent. Yet, a significant difference between the Veh and the ActD groups was found in the 24-h retention test (Figure 3C; $\chi^2 = 8.16$, df = 1, $p < 0.01$), thus revealing a significant impact of ActD for the memory retrieved at this time. In the 72-h retention test, the CS-specific memory was also significantly higher for the Veh than for the ActD group (Figure 3C; $\chi^2 = 4.55$, df = 1, $p < 0.05$).

These results thus reveal that a single conditioning trial induces, on the one hand, a 4-h memory that is translation but not transcription dependent, and on the other hand, a 24-h memory that is both translation and transcription dependent, contrary to what is usually affirmed. They also confirm that the 72-h memory depends on both transcription and translation, as previously described (Menzel and Müller, 1996; Menzel, 1999; Müller, 2013), even if such a memory was not expected after a single conditioning trial. Given that both the 24-h and the 72-h memories depend on transcription and translation, they should be referred as I-LTMs. On the contrary, if e-LTM refers to a memory that is translation dependent but transcription independent, it should be used to describe the 4-h memory.
Protein Synthesis Necessary for LTM Consolidation Is Still Ongoing 4 h after the Single Conditioning Trial

We showed that Eme injected 30 min before a single conditioning trial impairs subsequent LTM phases, which include in our case the 4-h, 24-h, and 72-h memories. To determine if the process of protein synthesis persists after training, we studied if Eme injection delivered 4 h after the single conditioning trial impairs memory retention at 24 h and/or 72 h post-conditioning (Figure 4A).

Veh-injected bees exhibited significant retention in the 24-h and the 72-h retention tests (Figure 4B), as their responses to the CS were significantly higher than to the NOd (24 h: $\chi^2 = 7$, df = 1, $p < 0.01$; 72 h: $\chi^2 = 3$, df = 1, $p = 0.0833$). Eme-injected bees responded more to the CS than to the NOd in the 24-h test but not in the 72-h test (24 h: $\chi^2 = 13$, df = 1, $p < 0.001$; 72 h: $\chi^2 = 8.07$, df = 1, $p < 0.01$). Eme-injected bees responded more to the CS than to the NOd in the 24-h test but not in the 72-h test (24 h: $\chi^2 = 1$, df = 1, $p < 0.01$; 72 h: $\chi^2 = 3$, df = 1, $p = 0.0833$).

The analysis of CS-specific responses revealed that the injection of Eme induced a significant decrease of CS-specific memory in the 24-h test with respect to Veh-injected bees (Figure 4C; $\chi^2 = 5.67$, df = 1, $p < 0.05$). The decrease of CS-specific memory observed in the 72-h test was not significant when compared to the Veh group ($\chi^2 = 0.754$, df = 1, $p = 0.385$). This result has to be considered with caution, as the performance of the Veh group was rather low and may thus have obscured significant differences with the Eme group. Taken together, these results suggest that 4 h after the single conditioning trial, a translation process is still present and is necessary for the consolidation of the memories retrievable 24 h post-conditioning.

Protein Synthesis Necessary for LTM Consolidation Is No Longer Present 7 h after the Single Conditioning Trial

We next studied the duration of the protein synthesis period induced by the single conditioning trial. We thus injected Eme 7 h after that trial and determined if this treatment impaired memory retention 24 h after conditioning (Figure 5A).

The population analysis showed that Veh- and Eme-injected bees exhibited significant memory retention in the 24-h retention test, as they both responded more to the CS than to the NOd (Figure 5B; Veh: $\chi^2 = 12$, df = 1, $p < 0.001$; Eme: $\chi^2 = 10$, df = 1, $p < 0.01$). An analysis of CS-specific responses confirmed that both groups did not differ significantly (Figure 5C; $\chi^2 = 0.0783$, df = 1, $p = 0.780$), thus showing that Eme injection 7 h after conditioning did not impair CS-specific memory at 24 h.

We conclude that 7 h after one-trial conditioning, the translation process required for the consolidation of the 24-h memory is already finalized. Furthermore, these results show that the effect of Eme injection is specific, as it is restricted to a finite temporal window.

Addressing a Multiple-Trial Conditioning Scenario

Even if addressing the topic of multiple-trial conditioning would require a separate study, we asked to what extent findings established in the case of multiple-trial conditioning are still valid under our experimental conditions. We determined if training bees with three conditioning trials spaced by 10 min leads to a protein-synthesis-dependent LTM, as stated by the established model of honeybee memory (Menzel, 1999, 2001, 2012). Bees were injected either with the Veh or Eme 30 min before conditioning (Figure 6A). Both groups of bees learned to respond to the CS in the same way (Figure 6B; two-way ANOVA for repeated measurements with Geisser-Greenhouse correction for sphericity; trials: F(1.814, 48.98) = 162, $p < 0.0001$; groups: F(1, 27) = 1, $p = 0.33$; trials x groups: F(1.755, 47.39) = 0.659, $p = 0.50$) and responded more to the CS than to the NOd 24 h after conditioning (Figure 6C; Veh: $\chi^2 = 21.0$, df: 1, $p < 0.001$; Eme: $\chi^2 = 13.1$, df: 1, $p < 0.001$). Yet, the group injected with Eme showed a significantly lower CS-specific memory, thus showing that Eme reduced memory expression 24 h after conditioning (Figure 6D; Pearson’s chi-square test; $\chi^2 = 6.84$, df: 1, $p < 0.01$). These results reproduce those of Stollhoff et al. (2005) (Figure 6E) and show that we are in a position to reproduce prior results referred to multiple-trial odor conditioning.
DISCUSSION

Extensive research on honeybee memory using the protocol of olfactory PER conditioning led to the established view that single trial conditioning has no capacity to induce long-term, olfactory memories that depend on protein synthesis (Menzel, 2012; Müller, 2012, 2013; Eisenhardt, 2014). Protein-synthesis-dependent memories have been typically circumscribed to protocols using multiple conditioning trials (Menzel, 1999, 2001, 2012). Indeed, multiple pairings of an odor and sucrose induce an e-LTM (~24 h post-conditioning) and an I-LTM (~72 h post-conditioning). These two forms of LTM depend on translation but differ in that only the I-LTM depends on transcription (Wüstenberg et al., 1998; Menzel, 1999).

However, inconsistent findings were sometimes reported even when multiple conditioning trials were used. For instance, in one work, e-LTM quantified 24 h after five spaced conditioning trials depended on transcription, as it was impaired by ActD injected shortly before conditioning (Menzel et al., 2001). This finding is nevertheless consistent with the scenario of our work, where a single conditioning trial induces a 24-h memory that depends on both translation and transcription.

Previous Reports on LTMs after One Trial of Olfactory PER Conditioning

Transient memories insensitive to protein synthesis inhibition have been described following one conditioning trial in olfactory PER conditioning. These memories span the range of seconds to minutes but were also reported for longer periods (Hammer and Menzel, 1995; Schwarzel and Müller, 2006; Menzel, 2012). The latter have received less attention, even if responses were recorded 1 or more days after conditioning (Sandoz et al., 1995; Grünbaum and Müller, 1998; Müller, 2000; Friedrich et al., 2004; Pamir et al., 2014). These memories were not integrated into the current model of honeybee memory and were considered independent of protein synthesis (Müller, 2012).

The molecular underpinnings of long-lasting memories induced by one conditioning trial were analyzed in four studies on olfactory PER conditioning (Grünbaum and Müller, 1998; Müller, 2000; Friedrich et al., 2004; Pamir et al., 2014). One of them (Müller, 2000) found that memory could be expressed from 1 to 4 days after a single conditioning trial (40% to 50% of bees responded) and that memory expression was insensitive to the blockade of the cAMP-dependent protein kinase (PKA). It was concluded that multiple trials are required to extend PKA activity as a condition for the formation of a protein-synthesis-dependent LTM. A similar conclusion was reached in a second study with respect to the necessity of a prolonged activity of the protein kinase C (PKC) for LTM (Grünbaum and Müller, 1998). In this case, one conditioning trial did not produce such an enhancement and led to a memory that could be retrieved from 1 to 4 days after a single conditioning trial (40% to 50% of bees responded) but was insensitive to transcription blockade by ActD (Grünbaum and Müller, 1998). Yet, recent analyses on the role of histone acetyltransferases (HATs) in honeybee olfactory memory contradicted this conclusion, at least for the 24-h memory, and support our findings (Merschbaecher et al., 2016).

In this study, inhibition of HATs by garcinol impaired a 24-h memory after a single conditioning trial, but the memory could be rescued by injection of ActD, thus indicating that the processes disturbed by garcinol require transcription.

In the two remaining works, the translation dependency of a 24-h memory induced by one conditioning trial was studied (Friedrich et al., 2004; Pamir et al., 2014). In both cases, Eme did not impair the 24-h memory, contrary to our findings. Yet, Eme was injected systemically (into the thorax), while we delivered directly into the brain (via the ocellar tract). Note that the same systemic thoracic injection was used in the study by Grünbaum and Müller (1998), where ActD proved to be ineffective. Both works (Friedrich et al., 2004; Pamir et al., 2014) used a concentration of Eme of 10 mM, which is half of the concentration used in our
work and that was ineffective to impair memory reconsolidation when injected after one retrieval trial, following three conditioning trials (Stollhoff et al., 2008). On the contrary, a concentration of Eme of 20 mM, similar to the one used in our experiments, was effective and impaired memory reconsolidation (Stollhoff et al., 2008).

It thus seems that part of the discrepancies about the molecular underpinnings of the long-lasting memories resulting from one trial conditioning are rooted in the different methods used to deliver the protein synthesis inhibitors and in their different doses. In addition, parametric differences between prior conditioning procedures and our experimental approach may also have contributed to the differences found.

**Parametric Differences between Our Experimental Procedures and Previous Works on Olfactory PER Conditioning**

What, then, are the main parametric differences between prior studies and our present work? First, contrary to many prior works, the bees used in our experiments were true foragers with high appetitive motivation, as they were captured at artificial feeders to which they were previously trained during foraging seasons (see STAR Methods). This is different from the traditional methodology of capturing bees at the hive entrance or even worse, from within the hive, without a proper control of appetitive motivation, which is crucial for an appetitive learning protocol. Second, for the same reason, we did not perform...
experiments during winter or bad-weather days or during periods of Asian-hornet predation in the summer. In addition, the overnight (ON) method described in the STAR Methods ensured the selection of the liveliest and most efficient learners. Third, our experiments made use of a novel olfactometer that provided a much better control of olfactory stimulation than did previous apparatuses used for olfactory bee conditioning (Szyiszka et al., 2014). This device ensured a precise control of the temporal properties of the CS and the NOd, excluded leakage and odor contaminations, and significantly reduced odor generalization, as shown by our preliminary experiments. In other words, it probably enhanced CS salience, thus favoring learning and retention (Rescorla and Wagner, 1972). This latter point is not trivial, as it leads to two further differences, which are conceptual rather than methodological. The fourth difference with many works done on honeybee memory is the use of a NOd in the retention tests to determine the specificity of the memories retrieved (Matsumoto et al., 2012). This point is important, as responses to the CS may include non-specific components, and it could be that only the specific components depend on protein synthesis. This hypothesis needs to be addressed experimentally in future works. Following our recommendations to include a NOd in memory tests (Matsumoto et al., 2012), experiments on olfactory learning in cockroaches showed that conclusions on 24-h memory varied if a NOd was omitted (Hosono et al., 2016). If only the CS were tested, memory was expressed after a single conditioning trial; yet, testing with a NOd revealed that this memory was not specific, as responses to the CS and to the NOd were indistinguishable (Hosono et al., 2016). This result underlines the necessity of including a NOd for assessing the specificity of memory. Finally, a fifth difference refers to this specificity and addresses a potential problem of PER conditioning protocols, namely, that population accounts of memory (% of bees responding to the CS)—the traditional and standard representation of memory in most works published—confound bees with and without specific memory. Indeed, given the binomial nature of PER responses (1 or 0), the percentage of CS responders may include bees with specific memory (which would respond only to the CS) and bees that would respond to the CS and to any other odorant. This problem was identified in analyses of learning performances, which found that the gradually increasing learning curve observed in many vertebrate learning paradigms reflects an artifact of group averaging (Gallistel et al., 2004). Similarly, population analyses of PER conditioning have been criticized, as they do not represent memory retention of individual honeybees (Pamir et al., 2011, 2014). This led us to perform an analysis of individual performances (bees with CS-specific memory) besides the traditional population account of memory. This analysis revealed that injecting Eme impaired retention at 4 h, 24 h, and 72 h post-conditioning, a fact partially hidden by the population analyses. A similar situation occurred in the case of ActD, which impaired the 24-h CS-specific memory, a fact that was concealed by the population account. This type of account, traditionally used for analyses of bee memory, may have therefore overlooked important features of honeybee memory.

The parametric aspects enounced here—particularly those related to the selection of motivated forager bees, season, and conditions for the experiments and the optimization of odor delivery—could have a significant impact on the molecular pathways activated by one conditioning trial. As mentioned above, multiple conditioning trials result in prolonged activation of PKA (Müller, 2000) and PKC (Grünbaum and Müller, 1998), which seems indispensable for LTM formation. Also, artificially enhancing processes that converge onto PKA activation such as nitric oxide (NO) signaling or the cyclic guanosine monophosphate (cGMP) promotes LTM formation (Müller, 2000). Interestingly, PKA levels vary with the bees’ satiation level: beesstarved for 18 h show a higher basal PKA activity in their brains than bees fed 4 h before (Friedrich et al., 2004). It is thus possible, given the specific control of feeding motivation achieved in our work compared to previous ones (see above), that individuals used in our experiments had PKA levels that facilitated LTM formation earlier and faster than expected. The same could occur with PKC levels and NO-cGMP signaling. If this were the case, the common procedure of collecting bees at the hive entrance, or even within the hive, may have resulted in mixed and uncontrolled variation of appetitive motivation and thus of PKA/PKC levels, which may have hidden the processes uncovered by our work. In addition, the traditional use of population response accounts instead of focusing on individual performances may have further hindered LTM detection in earlier post-conditioning periods.

The existence of two waves of transcription has been reported as a requirement for olfactory LTM formation in bees trained with multiple spaced conditioning trials (Lefer et al., 2012). The first wave would occur around the second conditioning trial and would be rather short (40 min), while the second wave would take place between 3 and 8 h post-conditioning. Whether the same two waves of transcription occur following a single conditioning trial remains to be determined. However, our results show that the injection of a transcription blocker before a single conditioning trial notably reduces the expression of the 24-h and 72-h LTMs. These results do not allow delimitating the temporal window of the transcriptional dependence but show its requirement. Moreover, Eme injection 30 min before the single conditioning trial, or 4 h (but not 7 h) after it, impaired the 24-h and 72-h LTMs, thus showing that the translation process required for LTM consolidation, be it unique or segregated in different waves, occurs within this period.

**LTMs Induced by a Single-Trial Conditioning across Species**

Our results allow reconciling long-standing discrepancies on appetitive memory formation with respect to the other most influential insect model in the field of memory studies: the fruit fly *Drosophila melanogaster*. In this insect, a single appetitive training session induces an LTM at 6 h and 22/24 h that requires de novo protein synthesis (Krashes and Waddell, 2008; Colomb et al., 2009; Trannoy et al., 2011). More generally, they are consistent with multiple lines of evidence showing that a single conditioning trial notably results in protein–synthesis-dependent memories in different species. For instance, in the snail *Lymnea stagnalis*, an LTM lasting for at least 21 days can be induced by a single appetitive conditioning trial (Alexander et al., 1984; Kemenes et al., 2006). This LTM depends on translation and transcription already 6 h after conditioning (Fulton et al., 2005). Also, in the ant *Formica fusca*, a single association of odor and sucrose in an exploratory
context induces a memory that persists until 72 h post-conditioning (but not longer) and that is sensitive to translation inhibition by cycloheximide (Piqueret et al., 2019). Parallels can also be found in the vertebrate literature. For instance, in adult rats, a single fear-conditioning trial in which animals learn the association between a tone and an electric shock leads to STMs and MTMs (the latter has not been characterized so far) and eventually, at longer delays, to a decaying memory that does not depend on protein synthesis. Adapted from Menzel (2012).

Situation reminds our findings with respect to the 4-h versus the 24-h and 72-h memories. Taken together, these results support the notion that a single conditioning trial has the capacity to induce different forms of protein-synthesis-dependent LTM. The 4-h memory depends on translation but not on transcription so that it has the characteristics of an e-LTM. The 24-h and the 72-h memories depend on both translation and transcription and should thus be considered as l-LTM. This model still mentions MTM for consistency with the previous model shown in (A), but note that MTM after one conditioning trial is still hypothetical and awaits characterization.

Conclusions

The picture that emerges from our study posits that a single PER conditioning trial is a salient learning experience that leads to LTM that are accessible 4 h later and that remain available 3 days after conditioning. These LTM depend on protein synthesis but differ in their dependency on translation and transcription processes (see Figure 7). The characterization of STMs and MTMs following one conditioning trial requires novel analyses addressing their molecular underpinnings. We thus conclude that the capacity of the bee brain to form protein-synthesis-dependent LTM based on unique experiences has been underestimated. Further studies should re-analyze the nature of olfactory memories arising after multiple-trial conditioning to provide a novel integrative perspective of memory in an insect that has played a pivotal role for our understanding of the biological bases of memory.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.01.086.

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AUTHOR CONTRIBUTIONS

M.E.V., P.M., H.V., and M.G. conceived the study. M.E.V. and P.M. designed, performed, and analyzed the experiments. M.E.V., P.M., and M.G. wrote the original draft. M.E.V., P.M., H.V., and M.G. edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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**STAR METHODS**

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse monoclonal anti-puromycin (clone 12D10) | Millipore | Cat# MABE343 |
| Mouse monoclonal anti-alpha tubulin-c (clone 12G10) | Drosophila Studies Hybridoma Bank | RRID: AB 1157911 |
| Goat Anti-mouse IgG, HRP conjugate | Sigma-Aldrich | RRID: AB 258426 |
| Chemicals, Peptides, and Recombinant Proteins |        |            |
| Emetine dihydrochloride | Sigma-Aldrich | CAS: 316-42-7 |
| Actinomycin D | Sigma-Aldrich | CAS: 50-76-0 |
| Phosphate Buffer Saline (PBS) | Euromedex | Cat# ET330 |
| Puromycin | Sigma-Aldrich | CAS: 58-58-2 |
| RIPA Buffer | Sigma-Aldrich | Cat# R0278 |
| Protease inhibitor cocktail | Sigma-Aldrich | Cat# P8340 |
| 1-Hexanol | Sigma-Aldrich | CAS: 111-27-3 |
| Nonanal | Sigma-Aldrich | CAS: 124-19-6 |
| Critical Commercial Assays |        |            |
| Western Lightning ECL pro | Perkin Elmer | NEL120E001EA |
| Software and Algorithms |        |            |
| ImageLabTM 6.0 | Bio-Rad | N/A |
| GraphPad Prism 8 | GraphPad Software inc. | N/A |
| Other |        |            |
| Bio-Rad Chemi Doc Touch | Bio-Rad | N/A |
| Hamilton syringe with a 34G needle | Hamilton | 85 RN SYR |

**LEAD CONTACT AND MATERIALS AVAILABILITY**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Martin Giurfa (martin.giurfa@univ-tlse3.fr).

This study did not generate new unique reagents.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Honeybees**

Honeybees were reared in outdoor hives at the experimental apiary of the CRCA situated in the campus of the University Paul Sabatier. In all cases, honeybee foragers (2 to 3-week old) were used. Maintenance of the hives was ensured by a full-time beekeeper, who used standard procedures for ensuring a healthy and active state of the colonies. No institutional permission is required for experimental research on honeybees.

**METHOD DETAILS**

**Animal preparation**

Bees were always collected one day before the training session. The bees used for the experiments were foragers collected at feeders filled with 40% sucrose to which they were previously trained. Captured bees were placed in boxes of 15 individuals where they received an average of 15 μl of 50% sucrose solution per bee (shared via trophallaxis). The boxes were kept overnight (ON) in an incubator at 28°C and 70% humidity. The mortality during this ON period was variable, ranging between 0 and 30% of the individuals. In the rare cases when the mortality exceeded 30%, all the bees were discarded. The ON method allowed homogenization of the bee’s satiation state inside each box due to trophallaxis. The ON method allowed us to select resistant bees that survived the night in the box, discarding weak bees as a source of poor performances. This method yielded a low mortality rate throughout experiments.
In the morning of the training day, the bees were cooled in ice and harnessed in plastic tubes secured with tape. Each bee was then fed with 5 μl of 50% sucrose solution.

**Conditioning protocol**

The training session was carried 3 hours after the feeding and consisted of a single conditioning trial (or three conditioning trials spaced by 10 minutes of intertrial intervals in the case of the experiment presented in Figure 6) in which an odorant was paired with sucrose solution (Matsumoto et al., 2012). The setup used for the experiment has been previously described (Szyszka et al., 2014). Briefly, CS delivery was provided by an automated odor-releasing machine (olfactometer) controlled by a microcomputer (Arduino® Uno). The harnessed bee was placed in front of the olfactometer, which released a continuous flow of clean air (3300 ml/min) to the bee antennae. Fifteen seconds after the onset of the training, the airflow was diverged upstream through the vial containing the odorant serving as the CS during 4 s. An air extractor was placed behind the bee to prevent odorant accumulation. The US was delivered manually to the antennae and proboscis for 3 s using a toothpick dipped into a 50% sucrose solution. The CS and the US had an overlap of 1 s. The bee was left in front of the clean air flow for additional 39 s, so that the training trial lasted 1 minute in total. The bees that did not respond to the sugar stimulation by extending their proboscis were excluded from the experiment.

The odorants used were 1-hexanol and nonanal (Sigma-Aldrich, France), which are perceived as dissimilar by bees (Guerrieri et al., 2005). Both were used pure and their role as conditioned stimulus (CS) or novel odorant (NOd) was balanced in a random way between bees in each experiment. A 50% sucrose solution (weight/weight) was used as US.

Retention tests were performed at different time points after the conditioning trial (1 h, 4 h, 24 h or 72 h post-conditioning). In a retention test, the CS was presented without reward; in addition, a novel odor (NOd) was also presented in order to assess generalization (when the CS was 1-hexanol, nonanal was the NOd and vice versa). Test odors were presented in a sequence that was randomized from bee to bee. Each test followed the same dynamics of the conditioning trial but with no reward delivery: the bee was placed in front of the air-flow for 15 s followed by 3 s of odor presentation, and then by 42 s without odor stimulation. The interval between the two odor tests was 10 min. The proboscis extension response to each odorant was measured. Each bee was tested in a single retention test, so that different groups of bees were used for the different retention tests. These groups were trained in the same day and tested at different time points. When the bees were tested 24 h or 72 h after training they were fed every afternoon with 10 μl and every morning with 5 μl of 50% sucrose solution to ensure their survival. Like for training, feeding occurred 3 - 4 hours before the test session.

At the end of the retention test, bees were tested for intact PER by touching their antennae with 50% sucrose solution. Bees that did not respond were excluded from the analysis (13%). We found no significant effect of emetine- or actinomycin D injection on sugar responses at any tested time.

Given the impossibility of training all groups at the same time, we decided to choose the 24-h group (Eme and Veh) as a reference and repeated it (Figures 2 and 3). In Figure 2, the groups done in parallel were 1 h versus 24 h, 4 h versus 24 h and finally 24 h versus 72 h; in Figure 3 the groups run in parallel were 4 h versus 24 h and 24 h versus 72 h. The repetition of the 24-h groups accounts for the differences in sample sizes between retention tests (Figures 2 and 3).

**Puromycin assay**

Bees were caught, harnessed and fed in the morning of the experiment. The median ocellus was removed and emetine (Eme, 0.2 μl, 20 mM in PBS, Sigma-Aldrich) or Phosphate Buffer (PBS, Euromedex) were administered 5, 30 or 60 min before the injection of puromycin (1 μl, 1000 μg/ml in PBS, Sigma-Aldrich). Both drugs were injected through the ocellar tract into the head capsule using a 5 μl Hamilton syringe (model: 85 RN SYR, needle size: 34G). Forty-five minutes after puromycin injection, the bees were anesthetized in ice, the head capsule was opened and the glands and trachea were removed. Each brain was extracted from the head capsule and immediately stored in ice in 125 μl of RIPA buffer with a protease inhibitors cocktail (120 μl of RIPA buffer (Sigma-Aldrich) and 4.8 μl of protease inhibitors (Sigma-Aldrich)) and homogenized mechanically. The homogenate of each brain was then centrifuged at 4 °C at 12000 rpm for 15 min. Part of the supernatant was stored at −20 °C and the rest was used to measure the protein concentrations using Bradford protocol.

Equal amounts (30 μg) of protein lysates of each sample were resolved by SDS-PAGE and electro-transferred to a nitrocellulose membrane. Blots were then briefly stained with Ponceau S to visualize total protein amounts and then subjected to western blot analysis with specific antibodies. Membranes were incubated in blocking buffer (5% BSA in PBS-Tw [0.1%]) for 2 h at room temperature and overnight with the primary antibodies anti-puromycin, clone 12D10 (1:5000, Millipore). They were washed with PBS-Tw [0.1%] and incubated with the secondary antibody (antimouse: Sigma-Aldrich) diluted 1:10000 in 1% BSA in PBS-Tw [0.1%] buffer for 1.5 h. After stripping of the membranes, the same procedure was repeated using primary antibody 12G10 anti-alpha tubulin-c (1:1000, Drosophila Studies Hybridoma Bank) diluted in 1% BSA in PBS-Tw [0.1%].

Protein bands were then visualized by chemoluminescence (Western Lightning ECL Pro, Perkin Elmer) using a Bio-Rad Chemi Doc Touch. Signals were quantified using ImageLab™ 6.0. Each lane was first normalized to its corresponding tubulin-c band. Then, for each membrane, the average value of the puromycin-only group was considered as a 100% signal, and all lanes normalized to it.
**Drug administrations**
Emetine 20 mM (Eme, emetine dihydrochloride, Sigma-Aldrich) was diluted in Phosphate Buffer (PBS, Euromedex). This concentration was chosen as it proved to be effective to impair memory assays while lower concentrations (e.g., 10 mM) are ineffective (Stollhoff et al., 2008). Actinomycin D [1.5 mM] (ActD, Sigma-Aldrich) was diluted in PBS. This concentration proved to be effective to study transcription processes in the bee brains (Lefer et al., 2012). The drugs were administrated 30 min before (Figures 2, 3, 6 and S1), 4 h after (Figure 4) or 7 hours after (Figure 5) the conditioning and/or 30 min before the 24-h memory retention test (Figure S1). They were delivered through the median ocellus, which was gently removed some minutes before injection. This method allows the drug to directly reach the brain via the ocellar tract (Tedjakumala et al., 2014). A volume of 0.2 μl was injected using a 5 μl Hamilton syringe (model 85 RN SYR) with a 34G needle.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

During learning and tests, a full extension of the proboscis in response to the CS was noted as a conditioned response (CR). Memory retention was computed at the population level as the percentage of responses to the CS and to the NOd. The specificity of memory was computed as the percentage of bees responding to the CS without responding to the NOd (CS-specific responses).

We assessed learning performances and differences in learning performances between groups by performing a repeated-measures two-way ANOVA (based on GLM) with the Geisser-Greenhouse correction for sphericity (Figure S2). The presence of memory for each group was assessed by comparing the responses to the CS and to the NOd (McNemar’s test). Differences in memory between groups were determined by comparing CS-specific memories (Pearson’s chi-square test or two-way ANOVA when more than 2 groups were compared; see Figure S1).

For each percentage, we calculated and represented the 95% confidence interval. The sample size (number of animals used) of each experiment is reported in the corresponding figure in parentheses. Statistical analyses were performed using GraphPad Prism 8 software.

**DATA AND CODE AVAILABILITY**

The datasets generated during this study are available at datadryad.org with the following accession ID https://doi.org/10.5061/dryad.bzkh1894z

This study did not generate any new code.