Parallel memory traces are built after an experience containing aversive and appetitive components in the crab *Neohelice*

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The neurobiology of learning and memory has been mainly studied by focusing on pure aversive or appetitive experiences. Here, we challenged this approach considering that real-life stimuli come normally associated with competing aversive and appetitive consequences and that interaction between conflicting information must be intrinsic part of the memory processes. We used *Neohelice* crabs, taking advantage of two well-described appetitive and aversive learning paradigms and combining them in a single training session to evaluate how this affects memory. We found that crabs build separate appetitive and aversive memories that compete during retrieval but not during acquisition. Which memory prevails depends on the balance between the strength of the unconditioned stimuli and on the motivational state of the animals. The results indicate that after a mix experience with appetitive and aversive consequences, parallel memories are established in a way that appetitive and aversive information is stored to be retrieved in an opportunistic manner.

long-term memory | consolidation | retrieval | appetitive | aversive

In the wild, it is crucial for animals to learn and remember which places represent a danger and which ones are associated with appetitive rewards such as food, shelter, or mate. Understanding how these associations are acquired, retained, and retrieved are major goals in neurobiology. A successful strategy suited to laboratory conditions has been simplifying learning episodes to appetitive or aversive experiences, because in those ideal cases, learning and memory can be unequivocally measured as attraction or avoidance. The present study is framed on the view that those pure appetitive or aversive experiences do not fully represent real-life situations. In nature, animals are exposed to stimuli that predict positive and negative consequences at the same time, and the conflicting information must be weighed and organized to allow expression of the most beneficial behavior. Invertebrates are convenient models to study the interaction between appetitive and aversive memory processes because neurochemical pathways and circuits involved in both types of memory have begun to be elucidated (1–5). The hypothesis that appetitive and aversive information interact during memory processes gets support from pharmacological studies in crabs (6, 7) and bees (8, 9), which show that the neurotransmitter necessary for appetitive memory formation does, in turn, impair aversive memory, whereas the transmitter necessary for aversive memory impairs appetitive memory. In addition, studies in *Drosophila*, in which memory mechanisms can be dissected at the neuron level, provide evidence that the interaction between appetitive and aversive information occurs from the circuits that encode reward and punishment to circuits that regulate memory expression (10–14).

In contrast to the knowledge about the interaction at the circuit level, the description about the consequence of competing experiences in regards to the content of memory is still sparse. Studies in honey bees have addressed memory after training in which the same odor is associated with simultaneous appetitive and aversive consequences (15, 16). The result that bees did not evidence appetitive memory toward the learned odor is interpreted as a consequence of an interaction between opposite reinforcements. However, if animals do not form any memory at all or if they form two opposite memories that compete during retrieval is not yet clear. A recent study in *Drosophila* indicates that appetitive and aversive memories are formed in parallel, and which memory prevails during memory expression depends on the time window in which memory is tested (17). Nevertheless, a limitation in these studies is that aversive and appetitive responses are mutually exclusive, which does not allow concluding whether the memory that is not observed does not exist or its expression is suppressed.

Here, we have addressed this question by using the crab *Neohelice* considering advantages that it offers for this study: It has two well-described appetitive and aversive memory paradigms (6, 18), both are contextual associative memories that use the same context as conditioned stimulus, and finally, appetitive and aversive memories become evident by two nonmutually exclusive behaviors. This last attribute allows that, if two different memories associated to the same context are formed, then they can be evidenced during the same test session. Crabs were subjected to a combined training protocol in which the conditioned context was associated with appetitive and aversive stimuli at the same time. We evaluated whether: (*i*) appetitive and aversive learning cancel each other in a way that no memory is formed; (*ii*) one learning rules over the other and, thus, only one memory is formed; (*iii*) animals make two independent associations and two separate memories are formed; (*iv*) opposite learning merge into a unique memory in which the conditioned stimulus adopt an intermediate valence.

Results

Weak, Low, Standard, and High Strength Training Protocols. We did initially a series of experiments aimed at establishing aversive and appetitive training protocols, which although they had opposite

Significance

In nature, animals are exposed to complex situations in which actions and stimuli predict appetitive and aversive consequences at the same time. To study memory formation after a learning episode that represents such real-life experience, we trained crabs in a context in which they found food while they were also threatened by a danger stimulus. We found that crabs build separate appetitive and aversive memories that compete during retrieval. Which memory is expressed depends on the strength of the unconditioned stimuli during training but also on the motivational state of the animal during retrieval. The results support that appetitive and aversive memories acquired during experience are independently stored to be used according to particular requirements during retrieval.

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valence, they were comparable in terms of their ability to form long-term memory. Thus, we systematically evaluated appetitive training protocols that varied in the amount of reward and aversive training protocols that varied in the number of presentations of the visual danger stimulus (VDS). One day after training, appetitive and aversive memory were tested. Fig. 1*A* shows the exploratory behavior of crabs that during training had been rewarded with different amounts of food. The untrained group (UT) was used to determine spontaneous level of exploratory behavior. No difference was caused by an appetitive reward of 20 mg of food, and it is therefore taken as a weak training protocol (UT vs. 20 mg: $F_{1,315} = 0.41$, $P = 0.98$). In contrast, rewards of 40, 80, and 160 mg of food induced higher exploratory behavior, which is taken as evidence of contextual appetitive memory and are therefore considered along the study as low, standard, and high strength appetitive training protocols (UT vs. 40 mg: $F_{1,315} = 3.05$, $P < 0.01$; UT vs. 80 mg: $F_{1,315} = 2.95$, $P < 0.05$; UT vs. 160 mg: $F_{1,315} = 3.01$, $P < 0.05$). Fig. 1*B* shows values of escape response elicited by the VDS after different intensities of aversive training. No difference in escape behavior is observed between untrained crabs and crabs that during training received five VDS presentations (UT vs. 5 VDS: $F_{1,315} = 0.58$, $P = 0.94$). In contrast, significant lower escape response, which is taken as evidence of aversive memory, is observed after training with 10, 15, and 30 VDS presentations and are therefore taken as low, standard, and high strength aversive training protocols (UT vs. 10 VDS: $F_{1,315} = 3.55$, $P < 0.01$; UT vs. 15 VDS: $F_{1,315} = 4.87$, $P < 0.001$; UT vs. 30 VDS: $F_{1,315} = 4.02$, $P < 0.001$).

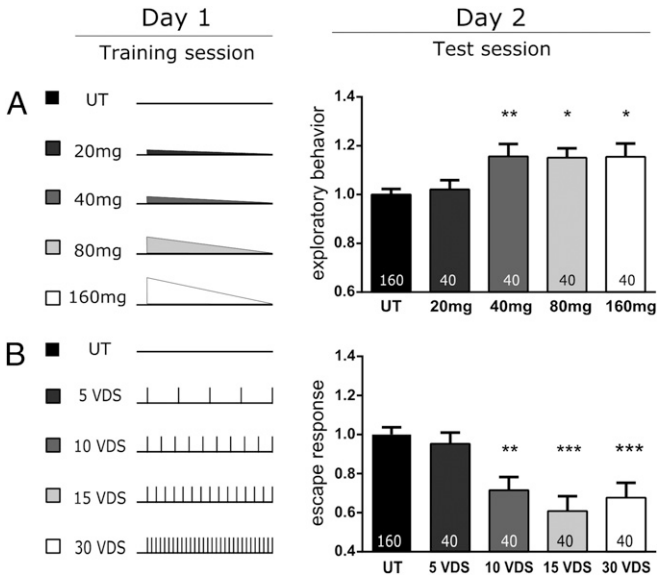

Fig. 1. Memory after appetitive and aversive trainings protocols of different training strength. (*A*) Appetitive training. Day 1 (scheme of the training session): UT, untrained group remains 50 min in the training context. AP groups, each animal receives a food pellet of 20, 40, 80, or 160 mg (weak, low, standard, and high training strength, respectively). Triangle size represents the amount of food consumed during the training session. Day 2 (test session): mean ± SEM of the exploratory activity normalized to the mean of the UT group. (*B*) Aversive training. Day 1 (scheme of the training session): UT, untrained group remains during 50 min in the training context. AV groups, each animal receives 5, 10, 15, or 30 VDS trials (weak, low, standard, and high training strength, respectively). Each vertical line represents one VDS trial. The duration of training is the same for all groups but the intertrial interval changes. Day 2 (test session): Mean ± SEM of escape response normalized to the mean of UT group. From black to white, grayscale indicates training strength from untrained group to high training strength group. $n = 40$ for all groups, except the UT groups which had $n = 160$. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ in Dunnet post hoc comparisons vs. UT group.

Simultaneous Appetitive and Aversive Training. In the next set of experiments, we combined aversive and appetitive training protocols as part of the same training session and explored how this combination affects appetitive and aversive long-term memory. All experiments had four groups of crabs (Fig. 2; day 1): one untrained group (UT), two groups that received only appetitive (AP) or aversive (AV) training, and one group called MIX, that during the 50 min that the crabs remained in the training context received the same amount of food as the AP group and the same number of VDS presentations as the AV group. One day after training (Fig. 2; day 2), aversive and appetitive memory was evaluated. Fig. 2*A* corresponds to the experiment in which two protocols of low training strength were combined. In agreement with the results shown in the first section, 40 mg of food and 10 VDS presentations induced long-term appetitive and aversive memory respectively (appetitive memory, AP vs. UT: $F_{1,156} = 2.01$, $P < 0.05$; aversive memory, AV vs. UT: $F_{1,156} = 2.06$, $P < 0.05$). However, neither appetitive nor aversive memory were evident in the MIX group although both trainings yield long-term memory when used alone (appetitive memory, MIX vs. AV: $F_{1,156} = 1.13$, $P = 0.26$; aversive memory, MIX vs. AP: $F_{1,156} = 0.87$, $P = 0.39$).

In the next two experiments, we increased the training protocols to standard and high training strengths. Appetitive and aversive memories were evident in the groups that received appetitive or aversive training respectively (Fig. 2*B*: appetitive memory, AP vs. UT: $F_{1,156} = 2.10$, $P < 0.05$; aversive memory, AV vs. UT: $F_{1,156} = 3.40$, $P < 0.001$; and Fig. 2*C*: appetitive memory, AP vs. UT: $F_{1,155} = 2.91$, $P < 0.01$; aversive memory, AV vs. UT: $F_{1,156} = 3.48$, $P < 0.001$). In contrast to the results in Fig. 2*A*, appetitive and aversive memory were evident in the MIX group after standard (appetitive memory, MIX vs. AV: $F_{1,156} = 4.14$, $P < 0.001$; aversive memory, MIX vs. AP: $F_{1,156} = 3.75$, $P < 0.001$) and after high training strength (appetitive memory, MIX vs. AV: $F_{1,155} = 1.99$, $P < 0.05$; appetitive memory, MIX vs. AP: $F_{1,155} = 2.89$, $P < 0.01$). Thus, we conclude that crabs can acquire and express long-term aversive and appetitive memories after a combined experience in which the aversive and the appetitive stimuli were presented associated to the same context during the same training session. However, when the training strength was just above the minimum protocol that is needed to induce long-term memory, then, no memory was observed in the MIX group, indicating a certain level of mutual interference.

Appetitive and Aversive Memories in the Same Animals. Based on the last two experiments, we concluded that after simultaneous appetitive and aversive training of high and standard training strength, crabs can form and express both memories. This interpretation is based on the average performance of the MIX group as a whole (Fig. 2 *B* and *C*). It might be possible that the performance of the MIX group is the result of two nonoverlapping subsets of animals: one that performed good in aversive memory and another that performed good in appetitive memory. This separation would indicate that both memories are mutually exclusive. To evaluate this possibility, we took the exploratory and the escape responses displayed by each individual crab during test and analyzed whether they were correlated in any way. We analyzed the experiments in which retention of both memories was observed after MIX training: standard (80 mg + 15 VDS; Fig. 3*A*) and high training strength (160 mg + 30 VDS; Fig. 3*B*). The scatterplots in Fig. 3 show all crabs in the UT (white circles) and the MIX groups (black circles) according to their individual exploratory behavior and escape response. No positive or negative correlation was found. The lack of correlation between both responses in the MIX groups suggests that appetitive and aversive memories are independent (Pearson correlation: $R^2 = 0.0466$, Fig. 3*A*; $R^2 = 0.0002$, Fig. 3*B*).

Next, we calculated what were the probabilities that a crab shows both memories after MIX training. For this analysis, we considered that the crab's behavior provides evidence of appetitive memory

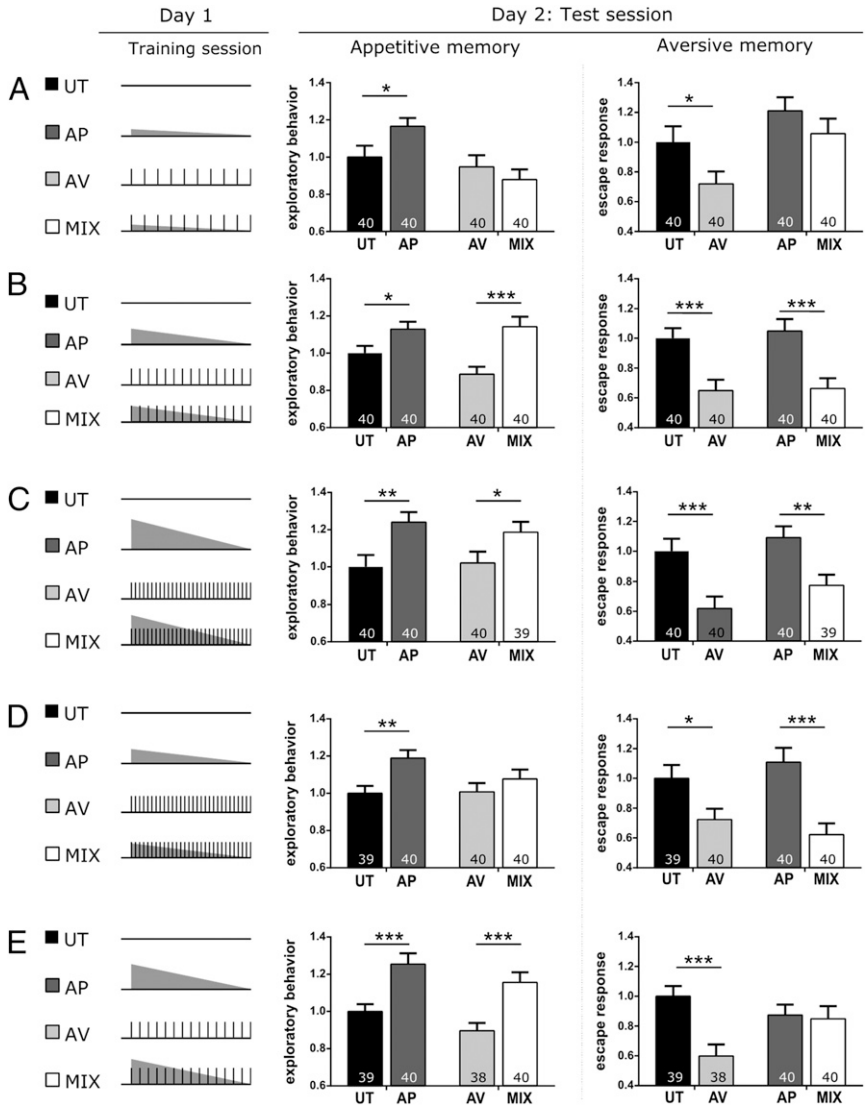

Fig. 2. Appetitive and aversive memories after simultaneous training. Labels: AP, appetitive trained group; AV, aversive trained group; MIX, appetitive and aversive trained group; UT, untrained group. Day 1, schemes of the training session; Day 2, appetitive and aversive memories measured as exploratory activity and escape response in the same animals. Values represent mean ± SEM of values normalized to mean response in the UT group. (*A*) Combination of low strength training protocols (40 mg of food and 10 VDS trials). (*B*) Combination of standard strength training protocols (80 mg and 15 VDS). (*C*) Combination of high strength training protocols (160 mg and 30 VDS). (*D*) Nonbalanced combination I: standard appetitive training and high strength aversive training (80 mg and 30 VDS). (*E*) Nonbalanced combination II: high strength appetitive training and standard strength aversive training (160 mg and 15 VDS). Black bars stand for UT groups, dark gray for AP groups, light gray for AV group, and white for MIX groups. Number of animals is indicated within each bar. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ in least squares comparisons between the indicated experimental group and its respective control.

when the exploratory activity elicited upon reexposure to the context is above the average exploratory activity measured in the untrained group. In Fig. 3, this criterion is met by crabs at the right half of the scatterplots (green and brown quadrants). In regard to aversive learning, we considered that the crab's behavior provides evidence of memory when the escape response elicited by the VDS is below the average escape response measured in the UT group. This criterion is met by crabs in the lower half of the scatterplots (orange and brown quadrants). The boundaries used to delimit the quadrants are defined by the mean responses of the UT group. The percent numbers in the corners of the figures indicate the percentage of crabs that after MIX training show evidence of only appetitive, only aversive, or both memories. Supported by the highest proportion of crabs in the "both memories" quadrant, we conclude that both memories can be formed and expressed by the same animal.

Finally, we analyzed whether the probability to evidence aversive memory after MIX training is the same as after only aversive training, and whether the probability to evidence appetitive memory after MIX training is the same as after only appetitive training. Fig. 3 *A* and *B*, show the percentage of animals that evidence appetitive or aversive memory. The appetitive memory bars include crabs in the green and brown quadrants, whereas the aversive memory bars include crabs in the orange and brown quadrants. The percentages of crabs evidencing memory in the AP and AV groups are shown in the right images, but for simplicity of the figure, data of individual crabs from these two groups is omitted in the scatterplots. Although there is no statistical difference between bars showing performance after MIX training vs. single training, we observed in all cases the trend that the percentage of crabs evidencing memory is slightly higher in the single training conditions, which may indicate a certain degree of mutual interference.

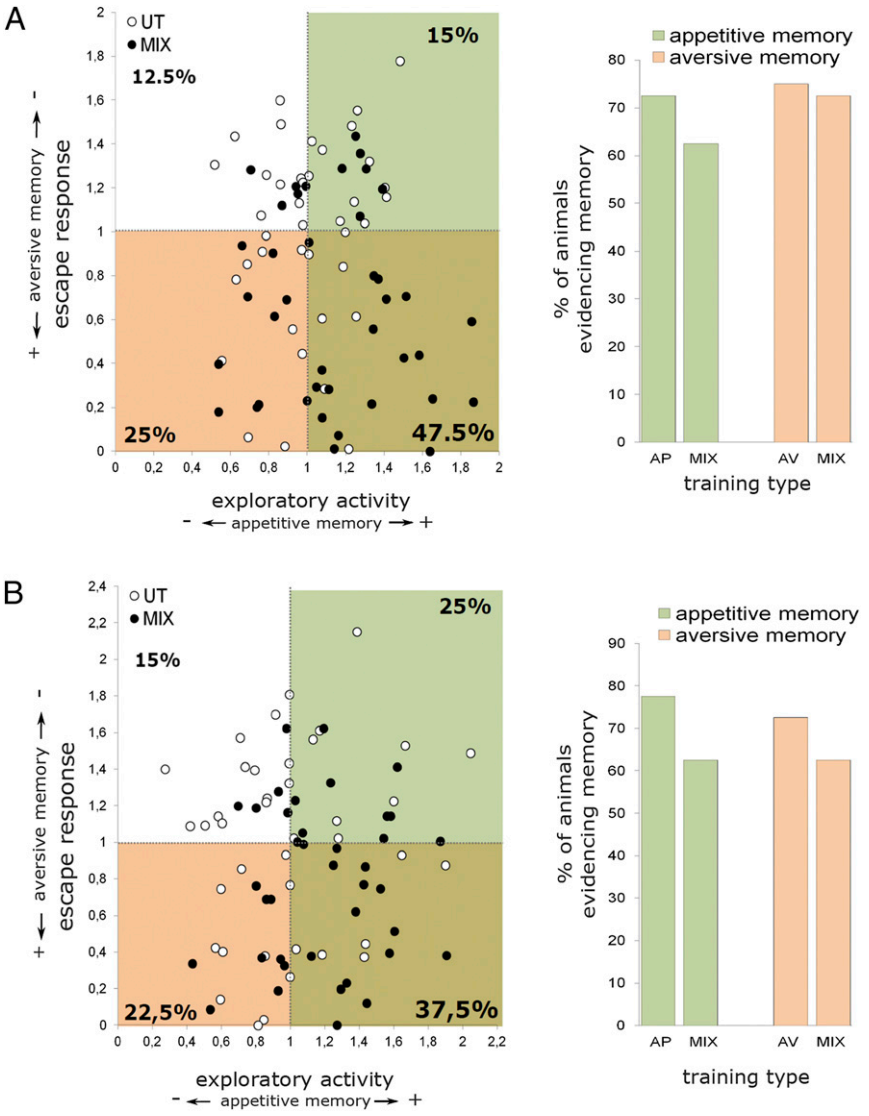

Fig. 3. Appetitive and aversive memories in the same animals. (*A*) Standard aversive and appetitive training protocols (same crabs as in Fig. 2*B*). (*B*) Strong aversive and appetitive training protocols (same crabs as in Fig. 2*C*). Each circle in the scatterplots corresponds to an individual crab described according to its exploratory and escape response. Open circles, UT; filled circles, MIX trained crabs. Quadrants are delimited by the mean responses of UT crabs. Crabs in the green quadrant are considered to evidence appetitive memory; in the orange quadrant are considered to evidence aversive memory, and in the brown quadrant are considered to evidence both memories. Numbers in the corners indicate the proportion of crabs from the MIX group in the quadrant. (*Right*) Appetitive memory (green bars): % of crabs in the AP and MIX groups with exploratory behavior above the mean response in the UT group; aversive memory (orange bars): % of crabs in the AV and MIX groups with escape response bellow the mean escape response in the UT group. No statistical difference within pairs of bars (χ^2).

Nonbalanced Appetitive and Aversive Training. Based on the results that interference between aversive and appetitive memory becomes evident depending on the combination of training strengths (Fig. 2 *A*–*C*), we tested memory after conditions in which appetitive and aversive trainings were intentionally not balanced. Fig. 2*D* shows the results after aversive training of 30 VDS (high training strength) and appetitive training of 80 mg of food (standard training). As expected, appetitive and aversive memories were clear in the AP and AV groups (Fig. 2*D*; appetitive memory AP vs. UT: $F_{1,155} = 2.98$, $P < 0.01$; aversive memory AV vs. UT: $F_{1,155} = 2.31$, $P < 0.05$). Interestingly, the MIX group showed aversive memory but not appetitive memory although appetitive training was of standard strength that yields memory when it is used alone or in combination with a standard aversive protocol (Fig. 2*D*; appetitive memory, MIX vs. AV: $F_{1,155} = 1.10$, $P = 0.27$; aversive memory, MIX vs. AP: $F_{1,155} = 4.11$, $P < 0.001$).

In a symmetrical experiment, we combined the high strength appetitive training (160 mg of food) and the standard aversive training (15 VDS). Appetitive and aversive memories are expressed in the AP and AV groups (Fig. 2*E*; appetitive memory, AP vs. UT: $F_{1,153} = 3.65$, $P < 0.001$; aversive memory, AV vs. UT: $F_{1,153} = 3.51$, $P < 0.001$). In reciprocity with the previous experiment, only appetitive memory was expressed in the MIX group, and no aversive memory was evident, although the crabs had undergone a standard aversive training protocol that yields memory when it is used alone or in combinations with a standard appetitive protocol (Fig. 2*E*; appetitive memory, MIX vs. AV: $F_{1,153} = 3.70$, $P < 0.001$; aversive memory, MIX vs. AP: $F_{1,153} = 0.24$, $P = 0.81$). These results demonstrate that either formation or expression of both memories are not completely independent from each other, because the efficiency of a given protocol to yield long-term memory is affected by the strength of the competing training protocol.

Separate Traces for Aversive and Appetitive Memories. Next, we focused again on the cases in which animals evidenced retention of both memories (Fig. 2 *B* and *C*). We wondered whether in those cases aversive and appetitive associations are integrated into a single memory trace, or alternatively, they are encoded as separate traces. To answer this question, we took advantage of the memory reconsolidation phenomenon well described in crabs (19, 20). Briefly, it has been shown that when a reminder is presented after memory has been consolidated, memory undergoes labilization and reconsolidation (21, 22). However, labilization only occurs when the reminder does not include the unconditioned stimulus (19, 23). We hypothesized that if after MIX training only one of the expected unconditioned stimuli (food or VDS) is presented during the reminder, then the memory that is linked to the unconditioned stimulus that is not presented can follow two possible fates depending on whether both memories are attached into a single trace or whether they are independently stored and retrieved. If both or no memory enter reconsolidation, it would mean that both associations are attached as part of the same trace. Alternatively, if the memory that was fulfilled during the reminder does not undergo reconsolidation and the one that was not fulfilled enters reconsolidation, it would favor the interpretation that both memories are reactivated as independent traces. Thus, we performed a series of experiments aimed at testing the independency of the appetitive and aversive memory traces during reactivation. Fig. 4 shows the experimental sequence and results. On the first day, crabs were trained by using the standard training protocols (80 mg + 15 VDS), thus obtaining the same four groups as in Fig. 2*B* (UT, AP, AV, MIX). On day 2, animals were injected with the protein synthesis inhibitor cicloheximide (CHX) and reexposed to the training context. During the reminder, all animals received 10 mg of food to fulfill the prediction of the appetitive memory but not the aversive memory. On the third day, animals were evaluated for appetitive and aversive memory. Fig. 4*A* shows retention of appetitive memory in the AP group (appetitive memory, AP vs. UT: $F_{1,146} = 3.44$, $P < 0.001$) but no aversive memory in the AV group (aversive memory, AV vs. UT: $F_{1,146} = 0.35$, $P = 0.73$). These results were expected because the reminder including food protects the appetitive memory from being disrupted by CHX. In contrast, aversive memory in the AV group entered labilization because of the incomplete reminder and, thus, CHX disrupted reconsolidation. Importantly, the MIX group behaved as the AP and the AV group because it showed normal appetitive memory (appetitive memory, MIX vs. AV: $F_{1,146} = 2.11$, $P < 0.05$) but no aversive memory (aversive memory, MIX vs. AP: $F_{1,146} = 0.83$, $P = 0.41$), which indicates that appetitive and aversive memory traces followed independent reactivation processes. To discard that the lack of aversive memory was due to ineffective aversive training, we ran in parallel two additional groups of crabs that were injected with vehicle instead of CHX: a control group (UT-saline) and an aversive trained group (AV-saline). The AV-saline group showed normal retention of aversive memory ($t_{78} = 2.02$, $P < 0.05$), confirming that the lack of aversive memory in the groups injected with CHX was not related with insufficient training or with the food received during the reminder, rather with disruption of reconsolidation.

In another experiment, we trained animals in the same manner as before, but, on day 2, the reminder included 1 VDS instead of food. Thirty minutes after the reminder animals were injected with CHX. On day 3, we observed aversive memory in the AV group (Fig. 4*B*; aversive memory, AV vs. UT: $F_{1,156} = 2.33$, $P < 0.05$) and no appetitive memory in the AP group (appetitive memory, AP vs. UT: $F_{1,156} = 1.24$, $P = 0.22$). Interestingly, the MIX group behaved again as the AV and AP groups, because it showed aversive memory but no appetitive memory (appetitive memory, MIX vs. AV: $F_{1,156} = 0.83$, $P = 0.41$; aversive memory, MIX vs. AP: $F_{1,156} = 2.43$, $P < 0.05$). Two additional groups, UT-saline and AP-saline, were treated in parallel and injected with vehicle instead of CHX to discard that the lack of appetitive memory was caused by insufficient

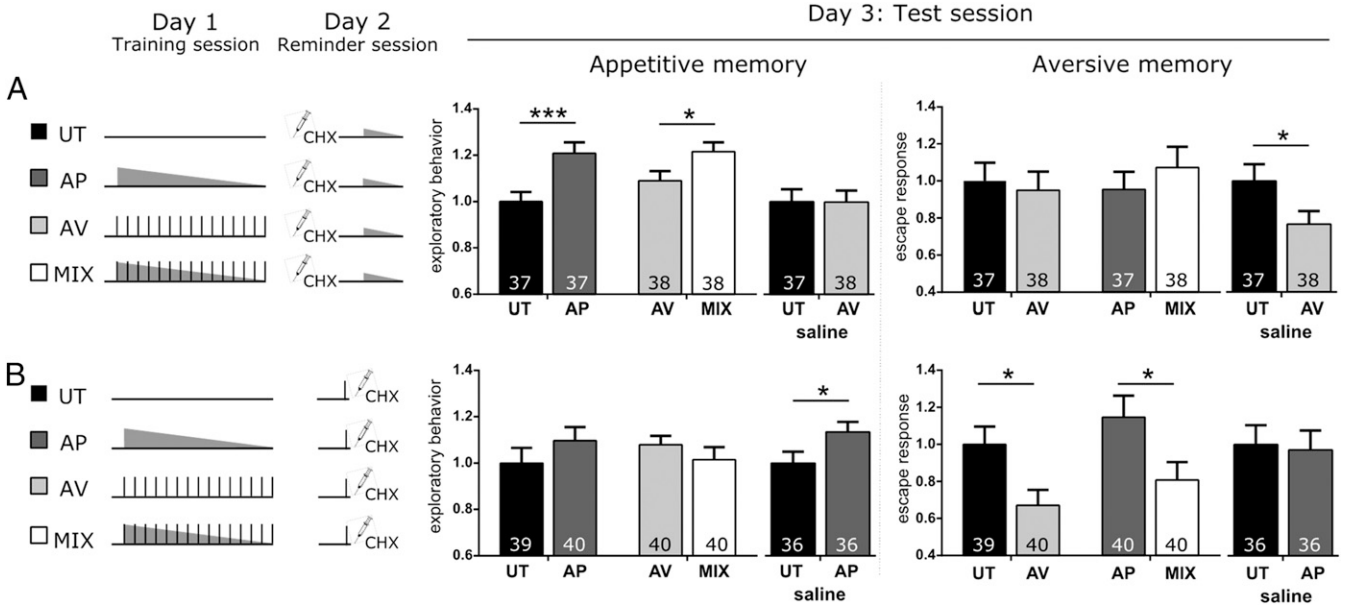

Fig. 4. Parallel appetitive and aversive memories are built after MIX training. Labels and symbols as in previous figures. Day 1: scheme of the training session. Day 2: scheme of the reminder session, CHX stands for cicloheximide (50 μg per animal). Day 3 test session: appetitive and aversive memories measured in the same animals. Values represent mean ± SEM of values normalized to mean response UT group. (*A*) Standard strength trainings (80 mg of food and 15 VDS). Reminder session: 10 min reexposure to the training context. Thirty minutes before the reminder, all animals are injected with CHX, except UT-saline and AV-saline groups, which were injected with saline. Five minutes after beginning the reminder, all animals receive a food pellet of 10 mg. (*B*) Standard strength trainings (80 mg of food and 15 VDS trials). Reminder session: 5 min reexposure to the training context. At the end of the reminder, all animals receive one VDS presentation. Thirty minutes after the reminder, all animals are injected with CHX, except UT-saline and AP-saline groups, which were injected with saline. $^*P < 0.05$, $^{***}P < 0.001$ in least squares comparisons between the indicated experimental group and its respective control.

appetitive training or to disruption caused by the VDS during the reminder. The AP-saline showed normal appetitive memory when it was compared with the UT-saline group ($t_{70} = 2.06$, $P < 0.05$).

In summary, only the memory that was not fulfilled during the reminder session could be disrupted by CHX, leaving unaffected the other one. These results are consistent with the interpretation that two parallel memory traces are established after MIX training and are independently activated upon reexposure to the training context.

Aversive and Appetitive Memories Interfere with Each Other During Retrieval. In this section, we wondered whether the interference between appetitive and aversive memories, as the cases are shown in Fig. 2 *A*, *D*, or *E*, takes place during acquisition or during retrieval of memory. To disambiguate between this two possibilities, we focused on the low strength MIX training protocol, after which appetitive and aversive memories were not evident in the MIX group (10 VDS + 40 mg of food) and used a reconsolidation protocol that can be used to disclose consolidated memory traces that are not expressed (24–27). The experiment shown in Fig. 5*A* was designed to evaluate the existence or the lack of appetitive memory after MIX training. On day 1, animals were trained by using the low strength training protocol. On day 2, crabs were placed in training context, and after 5 min, all of them received 1 VDS. After the reminder, all crabs were water deprived (WD) for 2 h. It has been shown in crabs that water deprivation around the time of reconsolidation enhances memory (19, 28). Finally, on day 3, crabs were tested for appetitive and aversive memory. As it was expected, AV and AP groups showed aversive and appetitive memory, respectively (Fig. 5*A*, *Right*; AP vs. UT: $F_{1,156} = 2.14$, $P < 0.05$; AV vs. UT: $F_{1,156} = 2.61$, $P < 0.01$). The MIX group, which did not evidence any memory when tested 1 d after training (Fig. 2*A*), disclosed appetitive but not aversive memory (appetitive memory, MIX vs. AV: $F_{1,156} = 2.21$, $P < 0.05$; aversive memory, MIX vs. AP: $F_{1,156} = 0.25$, $P = 0.80$), demonstrating that an appetitive memory trace is consolidated after MIX training. The observation that aversive memory was not enhanced was expected because it was not labilized by the reminder including the VDS.

Fig. 5*B* shows the complementary experiment designed to evidence the existence of the aversive memory trace after MIX training. On day 1, crabs were trained in the same manner as before, but on day 2, the context reminder included food instead of the VDS. On day 3, AP and AV groups showed appetitive and aversive memory, respectively (appetitive memory, AP vs. UT: $F_{1,152} = 2.58$, $P < 0.05$; aversive memory, AV vs. UT: $F_{1,152} = 4.40$, $P < 0.001$). In this experiment, the MIX group showed aversive but not appetitive memory (appetitive memory, MIX vs. AV: $F_{1,152} = 0.96$, $P = 0.34$; aversive memory, MIX vs. AP: $F_{1,152} = 3.51$, $P < 0.001$), demonstrating that an aversive memory trace was consolidated after MIX training.

In summary, these two experiments indicate that appetitive and aversive memory traces are consolidated after MIX training, suggesting that the mutual interference would take place during expression rather than during acquisition.

Motivational State Modulates Memory Expression. In the present section, we studied whether retrieval of aversive and appetitive memories are also modulated by factors that may change the relative weight of the aversive and appetitive memories after they have been consolidated. It has been shown in *Drosophila* that expression of appetitive memory, i.e., preference to go toward a rewarded odor, occurs only in hungry flies (29). Here, we designed and experiment to evaluate whether the state of satiation during retrieval influences memory expression in crabs. The crabs were trained following the same conditions as in the experiment shown as in Fig. 2*E*: strong appetitive training protocol (160 mg of food) and standard aversive training protocol (15 VDS). This combination yields

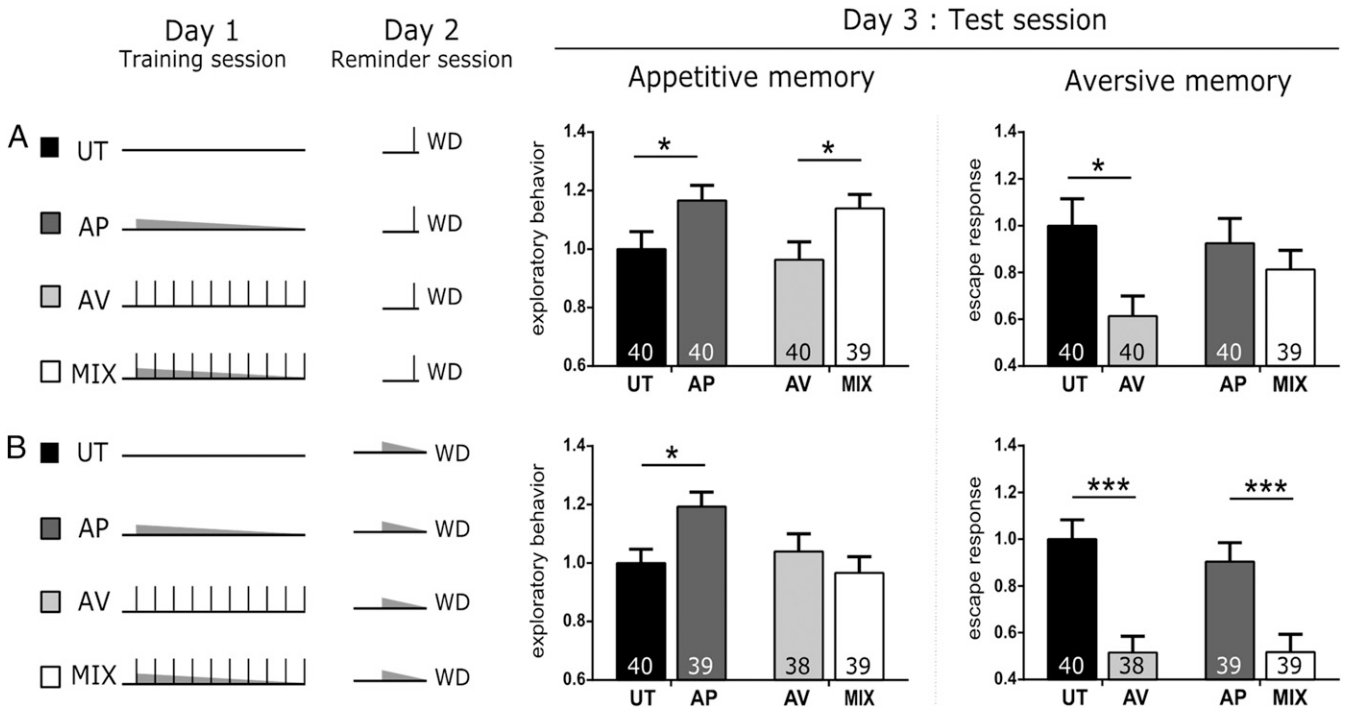

Fig. 5. Appetitive and aversive memories interact during retrieval. Labels as in previous figures. Day 1, scheme of the training session. Day 2 reminder session, WD stands for 2 h of water deprivation. Day 3, appetitive and aversive memories. Values represent mean ± SEM of values normalized to mean response of the respective UT group. (*A*) Low strength training protocols (40 mg of food and 10 VDS trials). Reminder session: 5 min re-exposure to the training context. At the end of the reminder all animals receive 1 VDS presentation and are water deprived for 2 h. (*B*) Low strength training protocols (40 mg of food and 10 VDS trials). Reminder session, 10 min reexposure to the training context. Five min after beginning of reminder, all animals receive a food pellet of 10 mg. After the reminder, animals are water deprived for 2 h. *$P < 0.05$, ***$P < 0.001$ in least squares comparisons between the indicated experimental group and its respective control.

expression of appetitive memory but not aversive memory. Six hours before the test session, the crabs were fed ad libitum in a container different to the resting container and the training context. Finally, all crabs were placed in training context and tested for aversive and appetitive memory. As observed in Fig. 6, despite crabs receiving a high strength appetitive training, neither the AP group nor the MIX group showed appetitive memory (appetitive memory, AP vs. UT: $F_{1,145} = 0.19$, $P = 0.85$; MIX vs. AV: $F_{1,145} = 1.11$, $P = 0.27$). To discard that the lack of appetitive memory was due to a failure in the appetitive learning, we carried out two additional AP and UT groups that were not fed before the test session. The "not-fed AP" group showed normal appetitive memory retention compared with its control ($t_{71} = 2.68$, $P < 0.01$), thus the no-expression of appetitive memory in the "fed-AP" and "fed-MIX" groups was a consequence of the pretest feeding. When we focused on aversive memory, we found memory expression in the AV group (aversive memory, AV vs. UT: $F_{1,145} = 2.95$, $P < 0.01$), which was expected after a standard training protocol, but also in the MIX group (aversive memory, MIX vs. AP: $F_{1,145} = 1.99$, $P < 0.05$), which was not expected based on the interference caused by the combination with the strong appetitive training (Fig. 2*E*). Thus, feeding the animals before testing had an inhibitory influence on appetitive memory that, in turn, facilitated expression of aversive memory, probably because of removal of the interference that retrieval of appetitive memory exerts on expression of aversive memory.

Discussion

In natural situations, animals are challenged to take decisions based on stimuli that predict competing aversive and appetitive consequences. In regards to learning and memory processes, it is not yet clear whether integration of competing information takes place during the encoding of the experience or during memory retrieval. To address this question, we simulated a real-life situation in which animals were exposed to a training context associated with a danger stimulus and an appetitive reward at the same time. Under this situation, we found that crabs built separate appetitive and aversive memories that are retrieved upon reexposure to the training context. Furthermore, interaction among the two memories with opposite valence becomes evident as a mutual interference during retrieval. We found that which memory prevails depends on the balance between the relative strength of the unconditioned stimuli and on the motivational state of the animals.

Formation and Expression of Two Memories. The result that allowed us to conclude that crabs establish appetitive and aversive memories after MIX training was that both memories were expressed during the same memory test session. This result was possible because the behaviors that evidence appetitive and aversive memories are not mutually exclusive. The behavioral output that makes evident contextual appetitive memory is an increase in the exploratory activity upon placement of the animal in the training context, whereas contextual aversive memory is revealed by a decrease in escape response elicited by the danger stimulus in the same context (6, 18). It is important to remark that although the aversive memory requires stimulation with the danger stimulus to be disclosed, the reactivation of it already occurs during reexposure to the training context at the same time that the appetitive memory is being expressed, as it is proved by the reconsolidation experiments. Notably, the aversive and appetitive memories associated to the same context is evidenced in a single memory test session. A limitation in previous studies using honey bees and flies was that expression of appetitive and aversive memories involves dichotomous decisions. In the T-maze used in flies to measure appetitive (30) and aversive memory (31), flies have to approach or avoid the arm containing the learned odor. When odors are associated with positive and negative rewards at the same time, the flies must decide between approaching or avoiding the odor, thus giving evidence of only one memory (17). In case of olfactory conditioning of proboscis extension in honey bees, the conditioned responses are either extending or withdrawing the proboscis upon stimulation with the odor (15, 32). Again, if the odor is associated with two competing consequences, the animal's behavior can provide evidence of only one memory. Thus, the results cannot be conclusive in regards to the existence or lack of the memory that is not expressed, because it may happen that the memory was not formed at all, that it was formed but is not retrieved, or that its expression is occluded by the competing behavior. From our results, we can unequivocally conclude that both memories were formed. Furthermore, the reconsolidation experiments that showed memory enhancement after water deprivation confirmed that both memory traces were consolidated, even when they were not evident during the test session (Figs. 2*A* and 5). Using reconsolidation, we also obtained evidence that the appetitive and the aversive components of the combined training protocol elicit the formation of parallel memory traces, in contrast to the idea that they are merged as elements of a single memory trace.

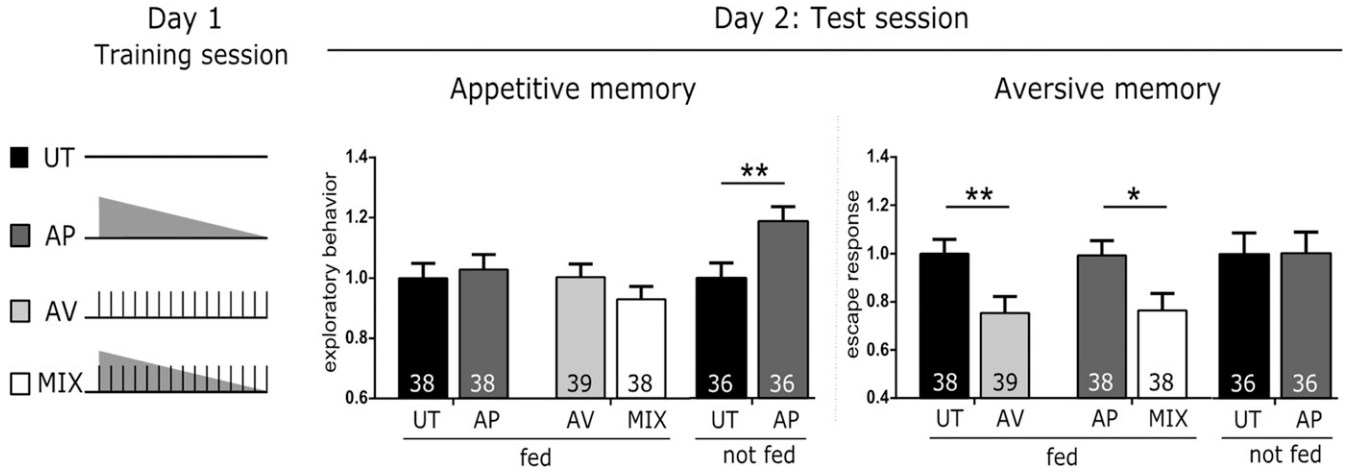

Fig. 6. Memory expression is modulated by motivational state. Labels as in previous figures. Day 1, scheme of the training session. High strength appetitive training and standard strength aversive training (160 mg and 15 VDS). Day 2, feeding and test session. All crabs received 320 mg of food 6 h before the test session, with the exception of crabs in the "UT not-fed" and "AP not-fed" groups. Values correspond to mean ± SEM of the exploratory and escape responses normalized to the mean of the respective UT group. $*P < 0.05$, $**P < 0.01$, in least squares comparisons between the indicated experimental group and its respective control.

Mutual Interference Between Appetitive and Aversive Memories. Memory impairment in MIX groups was more evident when training protocols were just above the minimum training strength necessary to determine expression of long-term memory (Fig. 2*A*), or when appetitive and aversive training were intentionally not balanced (Fig. 2 *D* and *E*). In the first experiment (Fig. 1), we observed that increasing training strength beyond the threshold to observe long-term memory did not endow more pronounced memory. However, the consequence of stronger training became clear in subsequent experiments analyzed in terms of the interference caused to or caused by the competing memory. Importantly, the fact that interference was observed by using two behaviors that are not mutually exclusive supports that the effect that we identify as memory interaction is not a conflict between two opposite motor commands, rather a modulation of the probability that a given memory takes control of behavior.

The next critical question was whether the interference between appetitive and aversive memory takes place during learning or during retrieval. The first hypothesis considered that each training weakens the formation of memory elicited by the opposite one, whereas the second hypothesis considered that both memories are fully formed as in the AP or AV groups, but they affect each other during retrieval. By using a memory reconsolidation protocol, we found that when no memory was observed in the MIX group, appetitive and aversive memory could still be enhanced during reconsolidation. If the interference between appetitive and aversive memories would have taken place during acquisition in a way that no memory was formed, then no memory could have been enhanced during reconsolidation. Thus, this result indicates that memory impairment in the MIX group is caused during expression rather than during learning or memory consolidation.

The brain areas and circuits involved in contextual aversive memory in crabs have just recently started to be elucidated (27, 33). Areas in the crab brain that are structurally homologous to the mushroom bodies of insects receive dopaminergic innervations and undergo plasticity related with the contextual aversive memory (7). In addition, it was shown that dopaminergic neurotransmission is necessary during aversive training to elicit formation of aversive long-term memory in crabs, as in honey bees and crickets (2, 3, 7, 16). We found that injection of dopamine coincident with appetitive training impairs formation of long-term appetitive memory in crabs and in honey bees (7, 9). In light of the present result, we interpret that dopamine injection emulates part of the effects that aversive training cause on appetitive memory. In regard to appetitive learning, octopamine is needed to elicit the formation of appetitive memory in crabs, honey bees, and crickets (1, 3, 6). Interestingly, injection of octopamine coincident with aversive training impairs formation of long-term aversive memory in crabs and honey bees (6, 8). Hence, we interpret that octopamine emulates part of the effects that appetitive training cause on aversive memory. Altogether, the results based on behavioral and pharmacological studies support the existence of tight crosstalk among aversive and appetitive pathways. Interestingly, a similar interaction was also found in Lymnaea between dopamine level, which signals appetitive rewards, and the ability of animals to form a conditioned taste avoidance memory (34).

The conclusion that interaction between competing appetitive and aversive information is intrinsic to the memory processes is also supported by studies in *Drosophila*. It was found that activation of neurons carrying information about appetitive stimuli inhibits the activation of neurons carrying information about aversive signals and vice versa (10, 12, 35). In agreement with our results, it was concluded based on behavioral results that after mixed training, flies built two parallel opposite memories that interact during retrieval. However, in contrast to our study, the two memory traces could be identified because they are expressed in different time windows after training (17).

Interestingly, it was shown in flies that instantaneous decisions taken on the basis of simultaneous stimuli that have opposite innate values involve the same mushroom body output neurons that participate on expression of appetitive and aversive memories (13). Thus, it is conceivable that opposite information acquired through experience is stored as independent memories that are integrated during retrieval according to the same rules used for stimuli with innate opposite values.

In the last experiment, we observed that the state of satiation modulates memory expression (Fig. 6). Feeding suppressed appetitive memory, as it was shown by studies in *Drosophila* (29), but more interesting was that expression of aversive memory was enhanced in satiated crabs. This result is consistent with recent findings in crayfish showing that feeding modulates risk avoidance behaviors (36) and with studies in Lymnaea showing that hungry animals suppress the expression of taste aversion memory (37). We interpret the results in crabs as the consequence of an evaluation that the animal does in relation to costs, benefits and risks that it is willing to take. This result gives special sense to the fact that memories are formed and stored independently until the moment of retrieval when the animal has to make a decision in regards to which one is the most convenient memory to be expressed. In summary, all presented results are consistent with the view that after an experience containing appetitive and aversive consequences, parallel memories are established in a way that learned information is available to be retrieved in an opportunistic manner.

Materials and Methods

Animals. Adult males *Neohelice granulata* crabs, 2.7–3.0 cm across carapace, were collected from narrow coastal inlets in San Clemente del Tuyú, Argentina. The crabs were maintained in a 12-h light/12-h dark cycle in collective tanks (20 animals per tank) filled to a depth of 2 cm with 12% artificial seawater prepared with hw-Marinex salt (pH 7.4–7.6; Winex). Experimental and holding rooms were kept between 22 and 24 °C. Experiments were carried out between 8 and 12 d after the animal's arrival to the laboratory, and each crab was used only in one experiment. Experiments were carried out in accordance with local regulations.

Experimental Design. Each experiment included one training and one test session. Some experiments included a reminder session on the day between the training and the test days. All sessions were carried out in consecutive days, thus the experiments lasted either 2 or 3 d, depending on whether they included a reminder session. Each experiment included either four or six groups of crabs, and each group of crabs had between 35 and 40 individuals. Appetitive and aversive training protocols were performed in the same training context that serves as conditioned stimulus in appetitive and aversive learning. The training and test context consisted in an opaque bowl-shaped container (12 cm high; 23 cm top diameter; 9 cm floor diameter) filled with artificial sea water to a depth of 0.5 cm. The crabs could freely move inside the container but were not able to escape from it. The container was illuminated from above by using a 10-W daylight lamp. The experimental room had 20 training/test containers separated from each other by walls, so that 20 crabs could be trained or tested independently and simultaneously. In each experiment, crabs and containers were assigned in a way that all experimental groups were run at the same time. The experimental scheme was repeated until reaching the final number of animals for the experiment. Exploratory activity and escape response were video-recorded at 2 or 10 Hz, respectively. Two days before the beginning of the experiment, each animal was marked with a little round piece 0.5 cm of yellow ethylene-vinyl acetate glued to the center of the carapace. Customized software was used to determine the *x-y* coordinates of the yellow spot, which allowed us to track the animals and calculate the distance covered by the animal during exploration and during escape intervals. Between sessions, animals were housed individually in opaque plastic cylinders filled to depth of 0.5 cm of artificial sea water in a separate room safe from external stimulations.

Appetitive Learning Paradigm.

Training session. The training protocol was the same as used before (6) with little modifications. Briefly, crabs were individually placed in the training context, and after 5 min, the trained group received a specific amount of food in the form of one rabbit pellet (Nutrientes Argentina SA). Normally, the crab explores the container until it finds the food pellet, when it starts eating it until the pellet is finished. Food quantity (the size of the pellet) in the trained groups

varied at 20, 40, 80, and 160 mg depending on the experiment. After 45 min, the crabs were removed from the training context and placed individually in boxes until the next session. The crabs in the untrained group remained in the training context without food during the 50 min that lasted the whole training session.

Reminder session. Twenty-four hours after training, animals were placed in the training context, and after 10 min, moved back to the resting container. It has been demonstrated in previous works that this short reexposure to the training context triggers labilization and reconsolidation of the memory formed the day before. Importantly, if the crabs receive a small amount of food (10 mg) during the reminder session, in a way that memory based predictions are fulfilled, then labilization and reconsolidation do not take place (19).

Test session. Twenty-four hours after training or after the reminder session, all crabs were placed in the training context and the exploratory activity was measured during 5 min.

Aversive Learning Paradigm.

Training session. The training context used in the aversive learning paradigm was the same one described for the appetitive learning paradigm. The VDS consisted in an opaque rectangular screen (25 × 7.5 cm) located 12 cm above the animal that was horizontally moved describing 90° excursion cycles. Each VDS trial had a total duration of 9 s during which the VDS moved two times over the animal. The crab´s displacements during the 9 s were measured as VDS-elicited escape response.

The aversive training protocol was similar to the one used before (38) with variation in the number of trials (5, 10, 15, or 30). The animals were placed in the training context, and after a 5-min period of adaptation, all crabs received one VDS trial. The response elicited by this first VDS trial was used to verify that groups did not differ in their responsiveness to the VDS. After that, the untrained group remained in the context during the whole training session with no further stimulation. The trained groups started their training 3 min after the first trial. Different numbers of trials were used to create different training strengths. The duration of training was the same in all cases, but the intertrial interval was adjusted to obtain a total training duration of 45 min in all groups. When the training was finished, all crabs were housed in the individual resting containers until the next session.

Reminder session. Twenty-four hours after training, the crabs were placed in the training context for 5 min. This short reexposure to the training context triggers labilization and reconsolidation of the memory formed the day before (20). However, if crabs receive a single presentation of the VDS during the reminder session, in a way that memory-based predictions are fulfilled, labilization and reconsolidation do not take place (23).

Test session. Twenty-four hours after training or after the reminder session, the crabs were placed in the training context and after 5 min, they received a VDS trial during which the escape response was measured.

MIX Aversive and Appetitive Training. MIX training consisted in simultaneous appetitive and aversive training. Crabs were placed in the training container, and after the 5-min period of adaptation, they had the initial VDS trial. Immediately after that, a pellet of food was deposited in the container. Three minutes later, the 45-min aversive training protocol started. When the training was finished, the crabs were housed in individual resting containers. For every animal, we have confirmed at the end of training that the pellet of food was consumed.

Statistical Analysis of Memory Expression. Memory retention was assessed by focusing analysis on test trial scores and looking for statistical differences between the response levels of the trained group and a corresponding control group (38). Rescorla (39) convincingly argued in favor of this type of analysis instead of a within-group comparison (training vs. testing) to distinguish between time of input (training session) and time of assessment (testing session).

Aversive memory. Operationally defined, a group of crabs that received aversive training is considered to express memory when its mean VDS-elicited escape response at the test trial is statistically lower than the mean escape response of a simultaneous control group, which had exactly the same manipulation and treatment with exception of the aversive training that is subject of analysis. Therefore, the statistical analysis was based on a priori LSD planned comparisons after a significant main effect in a one-way weighted means ANOVA with $\alpha = 0.05$ (per comparison error rate) (40, 41), except for the first experiment (Fig. 1), in which because there were four experimental groups and their performance was compared with a common control group, we used a Dunnet post hoc test after a significant main effect in a one-way ANOVA. When expression of aversive memory was evaluated in a group of crabs that had undergone only aversive training, the escape response was compared against the escape response measured in an untrained group of crabs (indicated along this study as the AV vs. UT comparison). Instead, when expression of aversive memory was evaluated in a group of crabs that had undergone MIX training, the comparison was made against a group of crabs that had undergone only appetitive training (indicated along this study as the MIX vs. AP comparison).

Appetitive memory. When a group of crabs has received food in the training context, contextual appetitive memory is disclosed 1 or 2 d later as a more intense exploration of the training context in comparison with a simultaneous control group that did not receive food in this context. Therefore, as for aversive memory, the statistical analysis was based on a priori LSD planned comparisons after a significant main effect in a one-way ANOVA, with the exception of the first experiment in which comparisons were made as indicated above for the aversive memory. When appetitive memory expression was assessed in a group of crabs that has undergone only appetitive training, the level of exploratory activity was contrasted against the activity measured in an untrained group (indicated along this study as the AP vs. UT comparison). Instead, when expression of appetitive memory was evaluated in a group of crabs that has undergone MIX training, the comparison was made against a group of crabs that had undergone only aversive training (indicated along this study as the MIX vs. AV comparison). By doing the analysis of the MIX group in this way, the only difference among the two groups is if they had or had not appetitive training on the first day. Although we consider this comparison to be the most appropriate to disclose aversive or appetitive memory in the MIX group, it is important to remark that comparisons of the MIX group against the UT group yielded in all cases similar results.

Amnesic and Hipermnesic Treatments. The protein synthesis inhibitor CHX (SIGMA-Aldrich C7698) was used in a 40 μg per animal dose diluted in 50 μL of crustacean saline solution (42) (molar concentrations: NaCl 0.45, $CaCl_2$ 0.015, $MgCl_2$ 0.021, KCl 0.01). Fifty microliters of saline or drug solution were injected through the right side of the dorsal cephalothoraxic-abdominal membrane by means of a syringe fitted with a sleeve to control the depth of penetration to 4 mm, thus ensuring that the injected solution was released in the pericardial sac. Because crabs lack of an endothelial blood–brain barrier (43) added to the fact that blood is distributed throughout a capillary system in the central nervous system (44) makes it possible to perform systemic injections that reach the neuropil areas of the brain.

Two hours of water deprivation after the reminder session was used as memory enhancement treatment during reconsolidation. Immediately after the end of reexposure to the training context, crabs were placed in their individual resting container without water for a period of 2 h. Water deprivation during consolidation or reconsolidation enhances both appetitive and aversive long-term memories (19, 28, 45).

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