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ORIGINAL PAPER

Contextual Pavlovian conditioning in the crab *Chasmagnathus*

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Abstract In contextual conditioning, a complex pattern of information is processed to associate the characteristics of a particular place with incentive or aversive reinforcements. This type of learning has been widely studied in mammals, but studies of other taxa are scarce. The context-signal memory (CSM) paradigm of the crab *Chasmagnathus* has been extensively used as a model of learning and memory. Although initially interpreted as habituation, some characteristics of contextual conditioning have been described. However, no anticipatory response has been detected for animals exposed to the training context. Thus, CSM could be interpreted either as an associative habituation or as contextual conditioning that occurs without a context-evoked anticipatory response. Here, we describe a training protocol developed for contextual Pavlovian conditioning (CPC). For each training trial, the context (conditioned stimulus, CS) was discretely presented and finished together with the unconditioned stimulus (US). In agreement with the CSM paradigm, a robust freezing response was acquired during the 15 training trials, and clear retention was found when tested with the US presentation after short (2 and 4 h) and long (1–4 days) delays. This CPC memory showed forward but not simultaneous presentation conditioning and was context specific and protein synthesis dependent. Additionally, a weak CPC memory was enhanced during consolidation. One day after training, CPC was extinguished by repeated CS presentation, while one presentation induced a memory labilisation–reconsolidation process. Finally, we found an anticipatory conditioned response (CR) during the CS presentation for both short-term (4 h) and long-term memory (24 h). These findings support the conditioning nature of the new paradigm.

Keywords Contextual conditioning · Consolidation · Reconsolidation · Extinction · Invertebrates

General introduction

The processing of contextual information is a key factor for adaptive behaviours. The association of incentive or aversive stimuli with different locations allows animals to make predictions that have a high adaptive value for displaying adequate responses. The recognition of particular locations involves complex processing of information from different sensory modalities that is stored together in a complete representation. This ability has been well characterised in mammals (Anagnostaras et al. 2001), for which the integrative and associative areas such as the medial temporal lobe, which includes the hippocampal formation, are in part devoted to contextual representation and recognition. It has been postulated that in insects, the mushroom bodies play a similar role in the integration of multimodal sensorial information and contextualisation (Liu et al. 1999; Strausfeld et al. 1998). In crustacea, contextual information processing

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In memoriam to our science mentor Héctor Maldonado. A true scientist who inspired us with his illimitable creativity.

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is necessary for the formation of contextual associative memory in the crab *Chasmagnathus granulatus* (recently classified as *Neohelice granulata*). In this contextual learning model, the repeated presentation of a visual danger stimulus (an opaque shape passing over the animal) provokes a decline in the escape response that had been elicited at the initial presentation of the stimulus (Lozada et al. 1990). Long-term memory (LTM) manifests at testing by a significantly lower response that is mainly due to the crabs' change in behavioural strategy from escape to a freezing response (Pereyra et al. 2000). It has been well described that massed training (e.g. 300 trials with 4-s inter-trial intervals) induces a non-associative habituation learning that does not show up in the first testing trial but only in subsequent trials, the so-called retraining phase of the testing session. Spaced training (e.g. at least 15 trials with 171-s inter-trial intervals) induces a context-dependent memory that is associative in nature, called context-signal memory (CSM), which appears at the first testing trial and at the retraining phase. However, considering the lack of change in the contextual cues over time, context capacity as a predictive stimulus is weak. Consequently, research on the anticipatory response that is dependent on the association has been hindered for 30 years.

A universal feature of long-lasting forms of memory is their dependence on macromolecular synthesis (Alberini 2008). LTM in *Chasmagnathus* has been found to be sensitive to the protein synthesis inhibitor cycloheximide during the first hours after training and to the mRNA synthesis inhibitor actinomycin D (Pedreira et al. 1995, 1996). Another general characteristic of LTM is that it can be enhanced during consolidation by the administration of drugs that increase excitatory neural activity. Bicuculline, an antagonist of $GABA_A$-type receptors, has previously been shown to induce the facilitation of LTM in *Chasmagnathus* (Carbó Tano et al. 2009).

Extinction of the context–signal association is obtained after prolonged re-exposure of the animals to the training context alone (typically for 2 h) one day after training. This process is sensitive to the administration of cycloheximide after the prolonged re-exposure (Pedreira and Maldonado 2003; Merlo and Romano 2008; Pérez-Cuesta and Maldonado 2009). Moreover, the reappearance of the original memory can be induced by recovery protocols (Hepp et al. 2010). However, after a brief (5 min) re-exposure to the training context without reinforcement, a reactivation process is induced that opens a new period of lability. Consequently, a re-stabilisation process begins in which protein synthesis is again required (Pedreira et al. 2002; Pedreira and Maldonado 2003; Merlo et al. 2005). NF-kB is a transcription factor that plays a key role in memory consolidation and reconsolidation (Meffert and Baltimore 2005; Romano et al. 2006b), and its role in *Chasmagnathus* memory has been characterised previously (Romano et al. 2006a). Sulfasalazine is a specific NF-kB inhibitor that acts on IkB kinase, which is the protein kinase that activates NF-kB. Sulfasalazine induces amnesic effects in *Chasmagnathus* for both consolidation (Merlo et al. 2002) and reconsolidation (Merlo et al. 2005).

Despite these well-described mechanisms that underlie the different phases of CSM, there is genuine doubt about its associative nature. As we mentioned earlier, in this CSM paradigm, the association between the iterated stimulus and contextual features is expressed not only in the first testing trial, but also in the retraining phase (Pedreira et al. 1998). That retention is expressed in the first trial suggests that the animals recognise the contextual features and thus execute the freezing response even during the first stimulus presentation. However, the context features are presented during the sessions in an unchanging way, which results in the context being a poor predictor. Consequently, the anticipatory freezing response is not found when the animals are exposed to the context. Thus, differences between the trained and control animals are observed only when the first negative stimulus is presented. It is also clear that in terms of Pavlovian conditioning, the anticipatory response is expected at testing when the conditioned stimulus (CS) is presented without the unconditioned stimulus (US). In Pavlovian contextual conditioning, the conditioned response occurs when animals are re-exposed to the training context. Consequently, the contextual associative learning of the crab could be interpreted as an associative habituation in terms of Wagner's theory (Whitlow and Wagner 1984) or as a non-typical conditioning in which learning is expressed only when the US is presented.

In the present work, we developed a training protocol for contextual Pavlovian conditioning (CPC) in which the features of the context were changed in a way that was contingent with the aversive stimulus, to create a predictor value for the US. Thus, for each training trial, the context (CS) was discretely presented and finished together with the US. Using such a protocol, we described consolidation, reconsolidation and extinction memory phases, and we tested for the presence of an anticipatory response that would be consistent with contextual Pavlovian conditioning. This new paradigm is useful in the search for the neuronal substrates of context representation because it allows for phasic presentations of the context (Sztarker and Tomsic 2011). Moreover, the adjustment of this new paradigm to the definition of Pavlovian conditioning allows comparative studies with paradigms that use mammals and may reveal shared components at a molecular level.

The first eight experiments were devoted to the characterisation of the different phases of LTM that were induced under this new training compared with the traditional protocol. In the usual training, the animals receive an above-light

illumination during the entire training session, while in the new training, the illumination changes from below to above during each trial. Thus, to compare the two training protocols, the testing for the new protocol was similar to the testing that has been used in the traditional training, in which the US is presented and the response to the US is analysed. It was predicted that the anticipatory response would be hindered by the conditions of the traditional training. Finally, in Experiment 9 and 10, the conditioned response (CR) was evaluated without the US presentation.

General materials and methods

Subjects

Adult male *Chasmagnathus granulatus* (*Neohelice granulata*, Crustacea, Grapsidae) inter-tidal crabs, 2.6–2.9 cm across the carapace, weight 17 ± 0.2 g ($n = 60$), were collected from water less than 1 m deep in the estuarine coasts of San Clemente del Tuyú, Argentina, and transported to the laboratory where they were lodged in plastic tanks (30 × 45 × 20 cm) filled to 0.5 cm depth with diluted (12 ‰, pH 7.4–7.6) marine water (prepared from Cristalsea Marinemix salts, USA) to a density of 20 crabs per tank. The holding room was maintained on a 12-h light–dark cycle (light on 07:00–19:00 hours). Temperature of both holding and experimental rooms was maintained within a range of 22–24 °C. Experiments were carried out between the third and the tenth day after the animals' arrival. Each crab was used in only one experiment. Experiments were carried out in accordance with the local regulations for the care and use of laboratory animals. All efforts were made to minimise animal suffering and to reduce the number of animals used. Furthermore, all the groups included the same number of animals in each experiment, 30 crabs. Consequently, considering that each experiment included four groups, the number of crabs used was 120.

Experimental device

The experimental device has been described in detail elsewhere (Romano et al. 1990). Briefly, the experimental unit was a bowl-shaped opaque container surrounded by a steep concave wall 12 cm high (23 cm top diameter and 9 cm floor diameter). The container was filled with marine water to a depth of 0.5 cm. The crab was placed in the container, which was suspended from an upper wooden framework (23 × 23 × 30 cm) by three strings. A motor-operated screen (US, an opaque rectangular strip of 25.0 × 7.5 cm) was moved horizontally over the animal from left to right, and vice versa. The screen's movements were cyclical. The screen displacements provoked the escape response of the crab and subsequent container vibrations. Each trial lasted 9 s and consisted of two successive cycles of movement. Four microphones were attached to the centre of the outside base of the container. The microphones recorded the vibrations that were produced by the animal's response. These signals were amplified, integrated during the entire trial (9 s) and translated into arbitrary numerical units ranging from zero to 8,000. During the experiment, the crabs were illuminated using a 5-W bulb placed either above or below the container (Pérez-Cuesta et al. 2007). A computer was employed to programme the trial sequences, trial illumination, trial duration and inter-trial intervals and to monitor the experimental events. The experimental room contained 40 experimental devices that were separated from each other by partitions.

The training and other treatment sessions were preceded by 10 min of adaptation to the experimental device, which was illuminated from below throughout. A standard CSM training session consisted of 15 US presentations without light shift (above illumination) with an inter-trial interval (ITI) of 171 s. A standard contextual Pavlovian conditioning (CPC) training session consisted of 15 trials. Each trial lasted 27 s with above illumination (CS), and the US was presented during the last 9 s. The ITI between US presentations was 171 s, as in the standard CSM protocol, but the ITI between CS presentations was 153 s. During the ITI, the experimental unit was illuminated from below, which provoked a virtual change in the environmental features. The untrained animals were kept in the experimental unit during the entire training session. These animals were not presented with the US but were presented with the same pattern of light shift. Immediately after each session, the crabs were moved from the experimental unit to individual resting containers, which were plastic boxes that were filled with water to a depth of 0.5 cm. The resting containers were kept inside dimly lit drawers. One trial of the US was presented before the training to measure the responsiveness of each animal. No differences were found between groups in this pre-training trial for any of the experiments.

Drugs and injection procedure

Crustacean saline solution (Hoeger and Florey 1989) or dimethyl sulphoxide (DMSO) was used as the drug vehicle (VHC), depending on which drug was used. Fifty microlitres of the VHC or drug solution was injected through the right side of the dorsal cephalothoracic–abdominal membrane via a syringe that was fitted with a sleeve to control the depth of penetration to 4 mm, thus ensuring that the injected solution was released into the pericardial sac. The following drugs were used: (+)-Bicuculline (BIC) (Fluka analytical), which is a competitive antagonist of $GABA_A$ receptor (Carbó Tano et al. 2009), was administered at a

final dose of 2.69 µg/g; sulfasalazine (SSZ) [2,4-hydroxy ((4-((2-pyridinylamino) sulphonyl) phenyl) azo) benzoic acid] (Sigma, St. Louis, MO, USA), which is an NF-kB inhibitor (Merlo et al. 2002), was freshly dissolved in crustacean saline with 10 mM HEPES pH 7.6 plus 15 % DMSO, for a final pH of 7.6 and a final dose of 0.5 µg/g; and cycloheximide (CHX), which is a protein synthesis inhibitor (Pedreira et al. 1995), was administered at a final dose of 2.35 µg/g.

Data analysis and drug effect evaluation

Retention of the learning acquired during training was considered to have occurred when a significantly lower level of response in the testing session was found for the trained group compared with its control group (i.e. both groups were injected with the same solution or treated with the same behavioural manipulation). The rationale for this criterion is based on the previous experiments performed in our laboratory. In these experiments, a significant difference (t test, $\alpha = 0.05$) between the trained (TR) and untrained (UT) groups was invariably identified at testing sessions that took place 24 h or more after training. The experiments demonstrating this difference included 15 or more training trials with 171-s inter-trial intervals. Accordingly, for the current experiment, a significant difference was predicted at testing between the UT and TR groups. Therefore, throughout the current paper, the results from the behavioural study were analysed using a priori planned comparisons via a weighted means ANOVA with α (per comparison error rate) = 0.05, according to the standard method (Howell 1987). A lack of difference between the UT and TR groups was assumed to indicate a lack of memory retention. For the case in which the extinction protocol was presented, a lack of retention was considered extinction memory. A comparison between the control groups that received different treatments was necessary to determine the possible drug or behavioural manipulation side effects that may have affected the response level at testing in a manner that was unrelated to training experience. In general, the statistical analysis of the test data included a set of three a priori planned comparisons, namely each pair of UT–TR groups and the comparison between the two UT groups, using planned comparisons of least squares means with α (per comparison error rate) <0.05 (Rosenthal and Rosnow 1985; Howell 1987). For each experiment, a prediction was made based on the experimental design. In general, we could predict that, in the first comparison, a difference between the UT and TR groups was expected due to the reduction in the response level that was induced by training in the latter group (e.g. this group received a strong training protocol without any other treatment). Conversely, in the second comparison, if the temporal relationship between the stimuli was changed or the drug impaired retention, then no difference was expected between the UT–TR pair. Finally, as long as the behavioural manipulation or the drug did not affect the level of response at testing, then no difference was expected in the comparison between control groups. All of the values were represented as the normalised mean ± the standard error with respect to the main control group (100 %, e.g. CPC, P or VHC).

Experiment 1: Pavlovian conditioning training induces robust retention

Initially, we developed a training protocol for CPC in which, for each training trial, the context (CS) was discretely presented and finished together with the US. A method that was used in previous research consisted of exposing the animals to the experimental device and, at a determined time point, changing the illumination only once from below to above, which was detected as a contextual change (Pérez-Cuesta and Maldonado 2009). Similarly, introducing the animals to the containers that were exclusively illuminated from below was not recognised as being similar to the training context, such that a 2-h exposure failed to induce memory extinction when the containers were illuminated from above during training (Hepp et al. 2010). In the current experiment, we induced a context shift during each training trial by changing the illumination from below (presented during the ITI) to above. Thus, the crabs were exposed to the above-light context alone for a few seconds before the aversive stimulus was presented during the last 9 s. Finally, the animals were exposed to a below-light context for another ITI.

Methods

The training session consisted of fifteen trials which were presented to half of the animals (the trained group, TR), while the other half received fifteen trials of light shifts without screen presentation (the untrained group, UT). One day after training, both groups were tested in six trials without the light shift and with the stimulus presentation (Fig. 1a). Using this type of testing permitted the associative component (first trial) and the signal-dependent component (retraining phase, 2–6 trials; Pedreira et al. 1998) to be analysed separately. We included a pair of UT and TR groups that were trained and tested using the standard procedure of continued above illumination to compare their retention levels with the retention obtained from the CPC protocol (Fig. 1a). Results at testing are presented as mean responses ± S.E.M. normalised with respect to the mean response of the correspondent UT group.

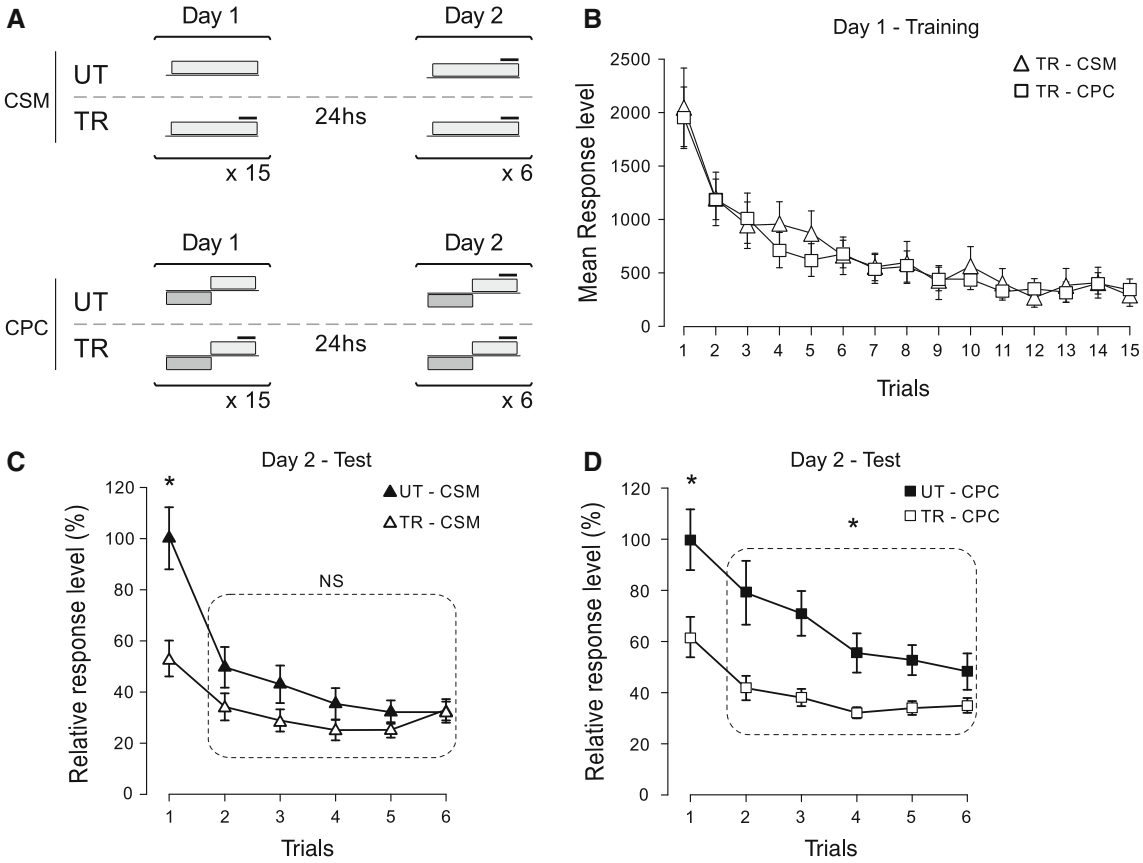

Fig. 1 Experiment 1. **a** Experimental protocols. The protocol for the CSM is presented in the *upper panel*. The protocol for the CPC is presented in the *lower panel*. The *black hyphen* at the end of *each box* represents one US trial. **b** Training curves for both trained groups for each trial. **c** Testing curves for the TR and UT groups for six trials of the CSM protocol. **d** Testing curves for the TR and UT groups for six trials of the CPC protocol. $^*P < 0.05$

Results and discussion

A repeated-measures ANOVA revealed no differences between the TR groups at training (Fig. 1b, $F_{1,78} = 0.05$; $P = 0.8$) and no group by trial interaction ($F_{14,109} = 0.4$; $P = 0.97$). However, differences were found between the first and last trial ($F_{14,109} = 31.43$; $P < 0.01$; E1-CSM vs. E15-CSM: $P < 0.001$ and E1-CPC vs. E15-CPC: $P < 0.001$). During the testing session, the TR groups showed a significantly lower level of response than the UT groups in both the standard and the CPC training during the first trial (Fig. 1c, d) ($F_{3,116} = 17.9$; $P < 0.05$; UT-CSM vs. TR-CSM: $P < 0.05$ and UT-CPC vs. TR-CPC: $P < 0.05$), which indicated that both protocols induced significant LTM. However, a difference was found between the two protocols in the retraining phase (trials 2–6). The CPC training resulted in significant differences between the UT and TR groups in the accumulated values block of trials 2–6 (Fig. 1c, d). Conversely, no difference was found between the UT and TR groups for the standard training protocol (Fig. 1d) ($F_{3,116} = 7.7$; $P < 0.05$; UT-CSM vs. TR-CSM: $P = 0.17$ and UT-CPC vs. TR-CPC: $P < 0.05$). The results from this section indicate that the CPC protocol induced strong LTM, which allowed the animals to show more memory retention than they would have shown from the usual training without contextual change.

Experiment 2: Pavlovian conditioning training induces a specific association between the context and the danger stimulus

The following two experiments were aimed at establishing whether the CPC training entailed an association between the contextual traits that were presented concomitant with the visual danger stimulus.

Methods

In the first experiment, we designed two pairs of groups: one group was trained using the CPC protocol (P, paired), whereas the other group was trained using the same light-shift pattern, but the aversive stimulus was presented before the above-light context (NP, non-paired). In this design, the

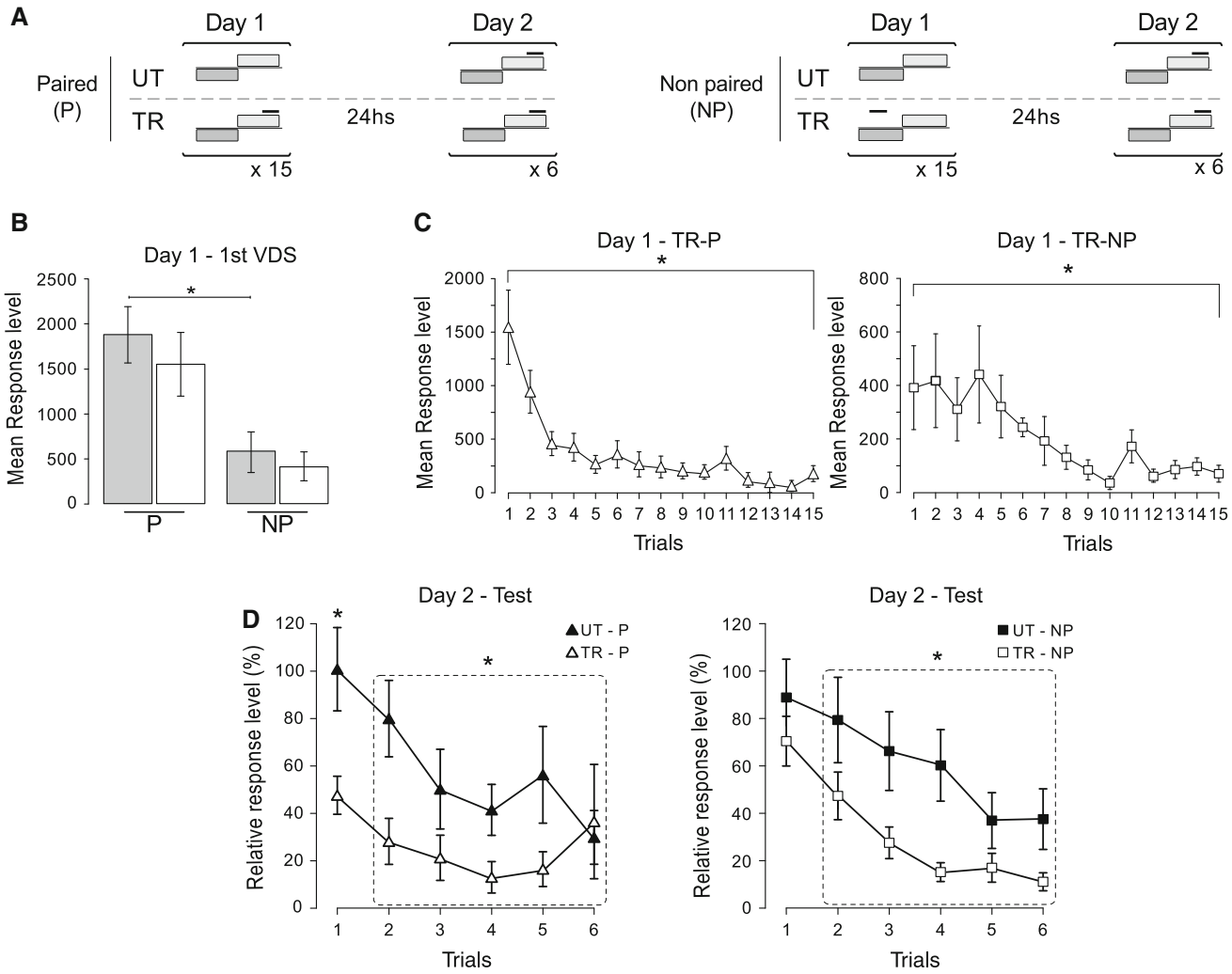

Fig. 2 Experiment 2. **a** Experimental protocols. The *left panel* represents the experimental protocol for the paired group (P). The *right panel* illustrates the experimental protocol for the non-paired group (NP), where the US is presented before the light change. **b** Mean response ± S.E.M. for the first US presentation for both UT–TR groups. *Grey bars* represent the UT groups, and *white bars* represent the TR groups. **c** Training responses. *Left panel* responses for the 15 training trials of the TR-P group. *Right panel* responses for the 15 training trials of the TR-NP group. **d** Testing session. Six testing trials are illustrated for the UT-P and TR-P groups in the *left panel* and for the UT-NP and TR-NP groups in the *right panel*. *$P < 0.05$

second pair of groups represented a strictly non-paired condition. Twenty-four hours after training, all of the groups were tested in six CPC trials (Fig. 2a). Results at testing are presented as in Experiment 1.

Results and discussion

During training, the first trial showed differences in the level of response between the paired and non-paired groups (Fig. 2b), with a significantly lower level of response from the non-paired groups than from the paired groups ($F_{3,116} = 3.36$; $P < 0.05$; UT-P vs. UT-NP: $P < 0.05$ and TR-P vs. TR-NP: $P = 0.002$). These differences may be attributable to the below-light condition making the US less salient for the non-paired groups. Despite the low level of response obtained from these groups, a learning acquisition curve was, nevertheless, detectable (Fig. 2c) ($F_{14,109} = 11.92$; $P < 0.01$; E1-P vs. E15-P: $P < 0.001$ and E1-NP vs. E15-P: $P = 0.02$). Furthermore, in an earlier report from our laboratory, Tomsic et al. (1991) demonstrated that these animals were able to form LTM without showing any type of measurable response under the effect of GABA that was administered before a 15-trial training session. The main conclusion from these experiments was that the execution of a response was not a necessary condition for acquisition. In the current experiment, the first trial of the testing session showed significant differences between the paired UT–TR groups, but no differences were found between the non-paired groups ($F_{3,116} = 3.36$; $P < 0.05$; UT-P vs. TR-P: $P < 0.05$ and UT-NP vs. TR-NP: $P = 0.25$). However, in

the retraining phase of testing, both pairs of groups showed significant differences (Fig. 2d) ($F_{3,116} = 3.46$; $P < 0.05$; UT-P vs. TR-P: $P < 0.05$ and UT-NP vs. TR-NP: $P < 0.05$). These results indicate that although both protocols induced retention during the retraining phase, only the paired condition was able to induce retention at the first testing trial. One possible interpretation for these results is that an association between the above-light context and the US could be established for this pair of groups and the presence of the CS primed the lower response to the US at the first testing trial. Conversely, in the non-paired group, the above-light context did not predict the presence of the US. Nevertheless, retention in the non-paired group was manifested during trials 2–6, which suggested that a non-associative component of this memory (habituation) was expressed as a faster reduction in the escape response over subsequent trials, which was indicative of re-learning or saving (Pedreira et al. 1998).

Experiment 3: CPC protocol: the importance of temporal relationship between the stimuli

For the CPC protocol, we used a forward conditioning design in which the CS was presented before the US and remained on until the US presentation finished. This design generally creates the best temporal relationship between the stimuli (Hall 1994). A less effective or ineffective protocol involves a synchronous onset for both stimuli, and then the CS remaining on alone for some time (Hawkins et al. 1986). To compare the effectiveness of both type of protocols, we performed the following experiment.

Methods

Two pairs of groups were included; one group was trained using the CPC protocol (TR-F; forward presentation), and the other group was trained using the same light shift but a different stimuli presentation schedule. For this second group, the US was presented during the first 9 s of the above-light context (TR-S; simultaneous presentation), and then the CS remained on alone for 18 s. Twenty-four hours after training, all of the groups were tested in six CPC trials (Fig. 3a). Results at testing are presented as in Experiment 1.

Results and discussion

During training, no differences between the TR-F and TR-S groups were found (Fig. 3b) ($F_{3,116} = 3.41$; $P < 0.05$). Differences were found between the first and last trial ($F_{14,109} = 38.08$; $P < 0.001$, E1-F vs. E15-F: $P < 0.001$ and E1-S vs. E15-S: $P < 0.001$). The absence of a difference between the two types of training may be explained by the independence of the intrasession diminution of the response level from the contextual cues and its exclusive dependence on the aversive stimulus (Suárez et al. 2010). For the first trial of the testing session, significant differences were found between the forward UT–TR pair, but no differences were found in the simultaneous onset pair comparison ($F_{3,116} = 3.41$; $P < 0.05$; UT-F vs. TR-F: $P < 0.05$ and UT-S vs. TR-S: $P = 0.24$). However, in the retraining phase of testing, both pairs of groups showed significant differences (Fig. 3c) ($F_{3,116} = 3.69$; $P < 0.05$; UT-F vs. TR-F: $P < 0.05$ and UT-S vs. TR-S: $P < 0.05$). Retention during the retraining phase is attributed to the non-associative component of the learning process (Maldonado et al. 1997). Therefore, only the forward condition was able to induce retention at the first testing trial, in which the associative component of the memory was expressed by itself. The forward protocol was more effective than the simultaneous protocol at establishing an association between the above-light context and the visual danger stimulus. Thus, the presence of the context before the US allowed the animals to perform the freezing response at the first testing trial. Conversely, the above-light context did not predict the presence of the visual danger stimulus for the simultaneous onset groups, and consequently, the crabs in this group performed the escape response at the first testing trial. In summary, the CPC paradigm shares the same temporal relationships as most conditioning tasks.

Experiment 4: Conditioning training is a contextual association and not a cue association

In the previous two experiments, we demonstrated that the paired but not the non-paired condition and the forward but not the simultaneous presentation of the stimuli induced long-term retention. These results support the establishment of an association between the above-light context and the visual danger stimulus. The light above appeared on its own in each trial and, during training, developed a high predictive value for the US because the light preceded the presentation of the US. For these circumstances, the light above could be interpreted as a conditioning cue. However, as we mentioned before, in the CSM paradigm, the animals establish an association between a multimodal context and the visual danger stimulus. It is also possible that in the CPC protocol, the conjunction of the context cues and the above light represents the CS. Therefore, to characterise the learning process that was generated by the CPC training protocol, we examined whether LTM was expressed only when evaluated in the presence of above-light illumination in the same training context or whether LTM can be generalised to other contexts with the same illumination. We performed the following experiment using the CPC training.

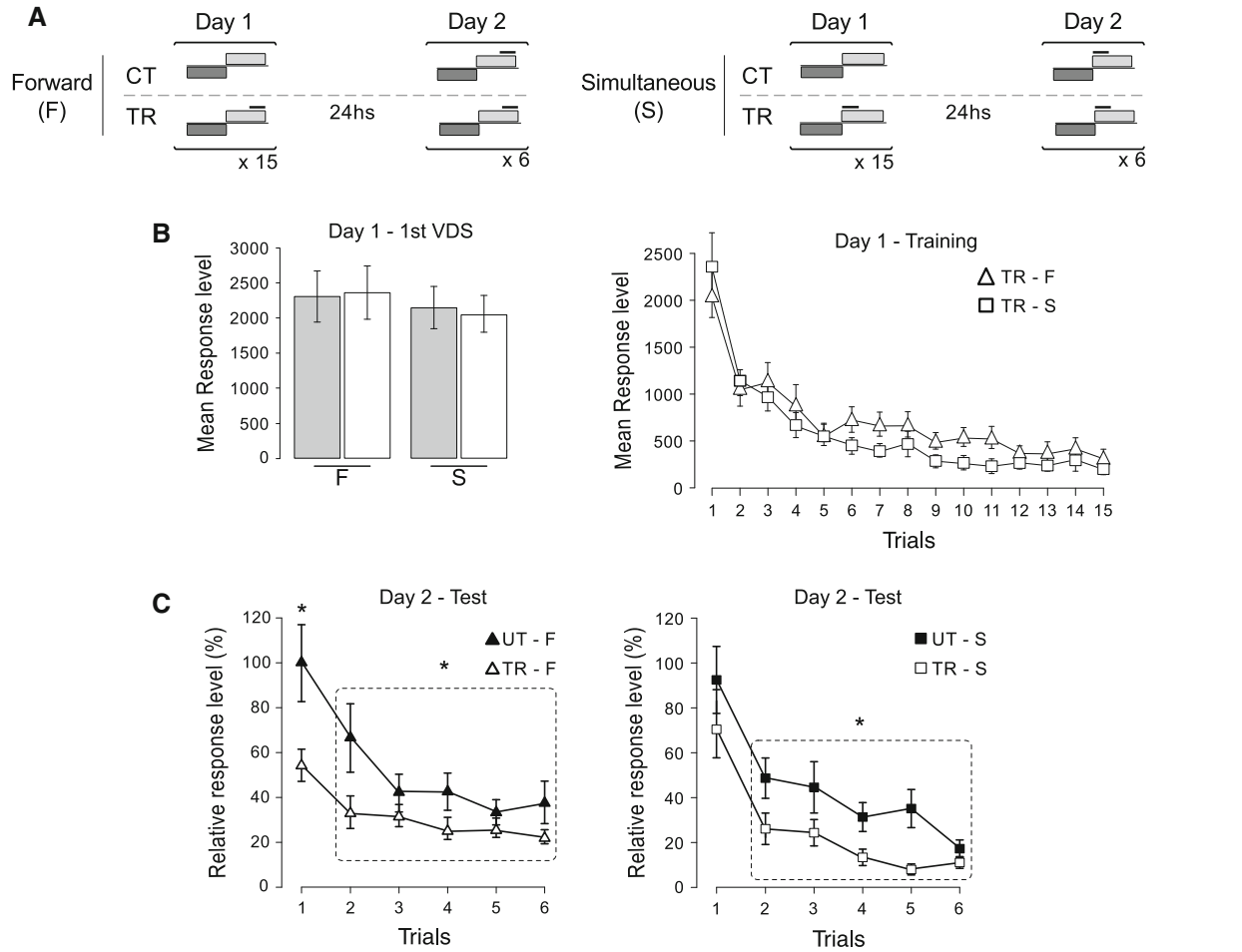

Fig. 3 Experiment 3. **a** Experimental protocols. *Left panel* represents the experimental protocol for the forward group (F). The *right panel* illustrates the experimental protocol for simultaneous presentation (S), where the US is presented in the first 9 s of the light change. **b** *Left panel* response of the first US presentation for both UT–TR groups. *Right panel* Training responses for the 15 training trials for both TR groups. **c** Testing session: Six testing trials are presented for UT-F and TR-F in the *left panel*; UT-S and TR-S in the *right panel*. *$P < 0.05$

Methods

Two pairs of UT–TR groups were formed. One pair was trained in the usual container device that had orange/blank walls (SC, same context), and the other pair was trained in a container that had white and black striped walls (DF, different context). These visual differences are salient enough to be differentiated by crabs under the usual training conditions (Tomsic et al. 1998; Pedreira et al. 2002). One day after training, all of the groups were tested in the blank container with six trials of visual danger stimulus presentation (Fig. 4a). Results at testing are presented as in Experiment 1.

Results and discussion

The results from the testing session are summarised in Fig. 4b. The UT–TR pair of groups that were trained and tested in the same context (orange/blank container, SC) showed significant differences in the first trial. These differences were due to the lower response displayed by the TR group compared with the UT group. Conversely, the UT–TR pair that was trained and tested in different contexts (trained in striped containers and tested in blank containers, DC) showed no differences between groups because the TR group displayed the same level of response as the UT group ($F_{3,116} = 3.89$; $P < 0.05$, UT-SC vs. TR-SC: $P < 0.05$ and UT-DC vs. TR-DC: $P = 0.89$). Moreover, the retraining phase showed the expected UT–TR differences for the SC groups and the absence of differences between the DC groups ($F_{3,116} = 3.69$; $P < 0.05$; UT-SC vs. TR-SC: $P < 0.05$ and UT-DC vs. TR-DC: $P = 0.2$).

Therefore, the results of the experiment indicate that CPC memory is context specific despite the identical illumination that was presented in both contexts. The lower level of response that was acquired during training was

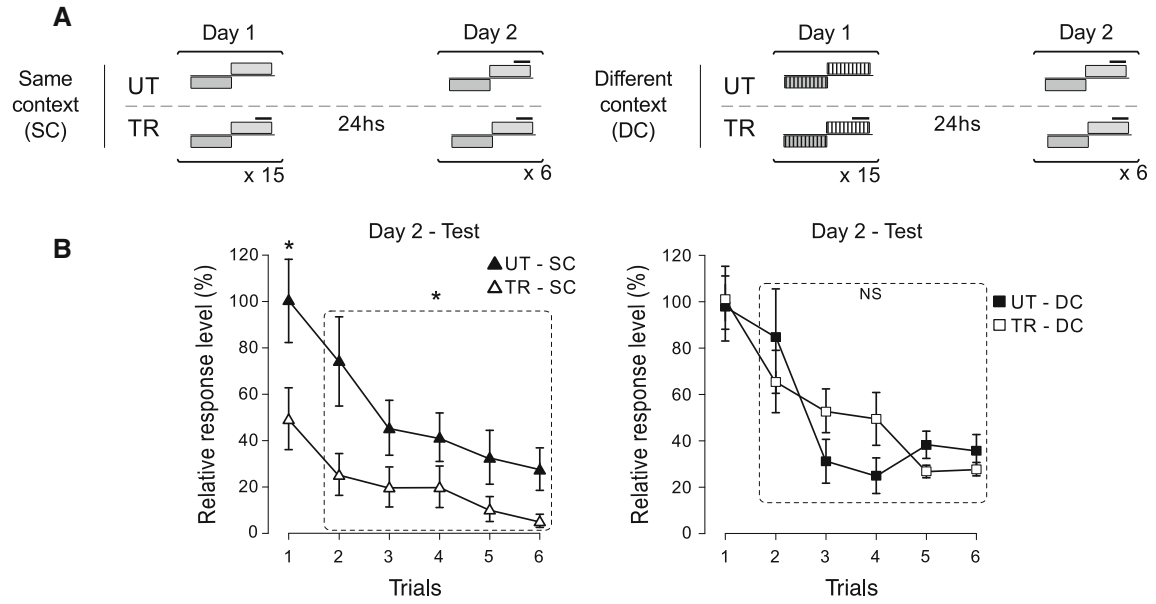

Fig. 4 Experiment 4. **a** Experimental protocols. The *left panel* represents the experimental protocol for the same context group (SC). The *right panel* shows the experimental protocol for the different context group (DC), where the training context (*boxes* with *vertical black stripes*) was different from the testing context. **b** Testing session: Six testing trials are represented for UT-SC and TR-SC in the *left panel*; UT-DC and TR-DC in the *right panel*. *$P < 0.05$

expressed only in the same context (orange/blank container), and the above-light illumination did not act as a cue on its own. Thus, in the CPC paradigm, the CS comprised the training context as a whole, which was composed of the contextual cues plus the light above. In this case, the above light could act as an occasion setter (Holland 1983; Brembs and Wiener 2006).

Experiment 5: Pavlovian conditioning memory undergoes extinction

Extinction is a universal characteristic of associative learning. For *Chasmagnathus*, extinction memory was found when the animals were exposed to the learning context 24 h after training. The extinction memory was induced using either continuous or discrete context presentations that occurred for at least 2 h but not for shorter periods (Pedreira and Maldonado 2003; Hepp et al. 2010). This new memory was temporary in nature. As time passed, spontaneous recovery of the original memory typically occurred (Merlo and Romano 2008; Hepp et al. 2010). Taking into account the structure of the CS in this new paradigm, we opted to use an extinction protocol that consisted of light-shift trials. The total re-exposure time was 2 h.

Methods

Two pairs of UT and TR groups received the CPC training on Day 1. One day later, one pair of groups remained in a different context for 2 h (NoE, no extinction), and the other pair received an extinction session (E, extinction) which consisted of 15 trials of light shift presented in a way that ensured 2 h of above-light exposure (E) (Fig. 5a). Results at testing are presented as in Experiment 1.

Results and discussion

The one-trial testing session showed retention only in the non-extinction UT–TR pair groups (Fig. 5b) ($F_{3,116} = 3.18$; $P < 0.05$; UT-NoE vs. TR-NoE: $P < 0.05$ and UT-E vs. TR-E2: $P = 0.32$), which indicates that the 2-h session was effective at inducing extinction. To confirm that the lack of retention was due to an extinction process, we evaluated the existence of spontaneous recovery by retesting the crabs 48 h later with six trials. Spontaneous recovery has been described previously in studies of the standard CSM protocol (Merlo and Romano 2008; Hepp et al. 2010). In the current study, memory retention was found for both pairs of groups (Fig. 5b) ($F_{3,116} = 3.36$; $P < 0.05$; UT-NoE vs. TR-NoE: $P < 0.05$ and UT-E2 vs. TR-E2: $P < 0.05$), indicating that extinction decayed during these 2 days and that the original memory was expressed again.

Experiment 6: Memory consolidation from Pavlovian conditioning is dependent on protein synthesis

An almost universal feature of enduring memories that persist for at least a day is their dependence on protein synthesis

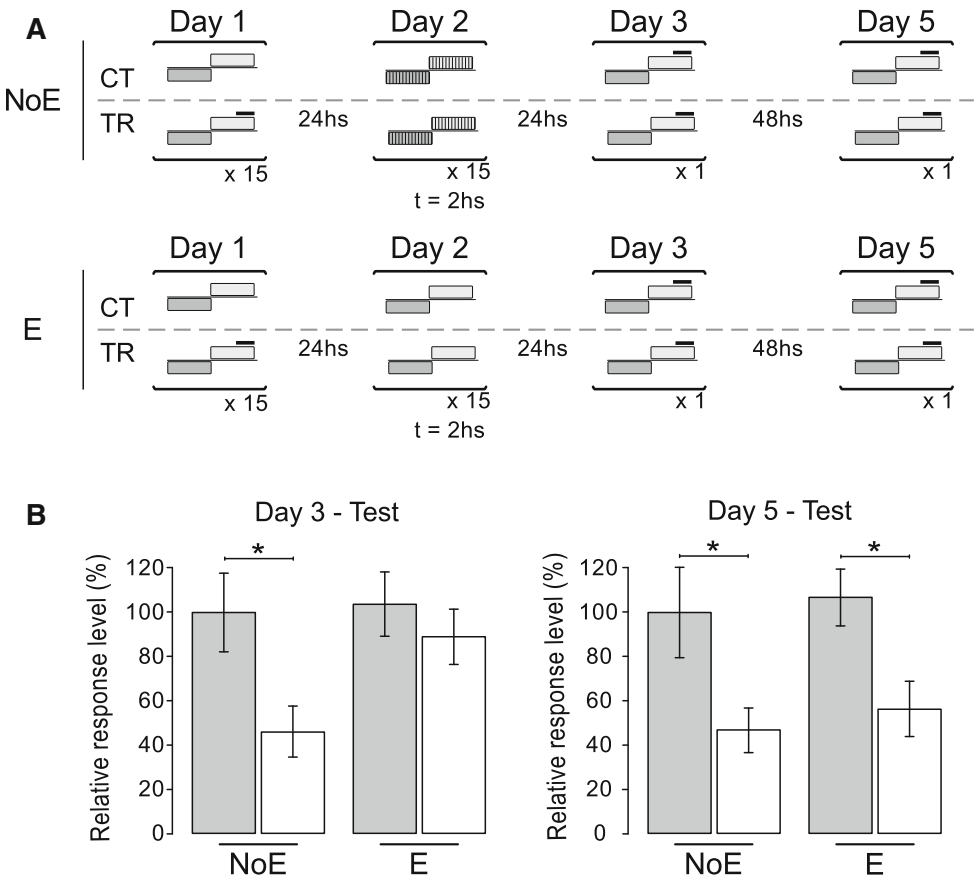

Fig. 5 Experiment 5. **a** The *upper panel* represents the experimental protocol for the no-extinction control group (NoE), and the *lower panel* illustrates the experimental protocol for extinction group (E). Day 1: training session of 15 US presentations. Day 2: No extinction session: animals were exposed to a different context (*boxes* with *vertical black stripes*) than the context they had been exposed to on Day 1; extinction session: animals were re-exposed to the same context as on Day 1 ensuring 2 h of context re-exposition. Day 3: test session (one trial). Day 5: test session (one trial). **b** Testing session. *Grey bars* stand for UT groups and *white* for TR groups. *$P < 0.05$

during consolidation. To test whether the CPC memory was dependent on general protein synthesis, we performed an experiment using two pairs of UT–TR groups.

Methods

One pair of groups was injected with VHC after the CPC training (VHC), and the other pair was injected with 40 µg/animal of the translation inhibitor CHX (CHX). This dosage is commonly used in our laboratory to induce amnesic effects (Pérez-Cuesta and Maldonado 2009). One day after training, the animals were tested in one testing trial, as is usually performed in pharmacological experiments (Fig. 6a). Results at testing are presented as in Experiment 1.

Results and discussion

As expected, the UT vs. TR comparison for the vehicle-injected pair yielded significant differences between groups, which indicated memory retention. Conversely, the cycloheximide-injected pair showed no significant differences between groups, which indicated an amnesic effect (Fig. 6b) ($F_{3,116} = 3.93$; $P < 0.05$; UT-VHC vs. TR-VHC: $P < 0.05$ and UT-CHX vs. TR-CHX: $P = 0.52$).

Experiment 7: Memory consolidation from Pavlovian conditioning is enhanced by a $GABA_A$ receptor antagonist

Another general characteristic of LTM is that it can be enhanced during consolidation by the administration of drugs that increase excitatory neural activity. Previous research has found that bicuculline, an antagonist of $GABA_A$-like receptors, induces the facilitation of LTM in *Chasmagnathus* (Carbó Tano et al. 2009).

Methods

In the present experiment, we administered 2.69 µg/g of bicuculline to a pair of UT–TR groups (BIC), while another

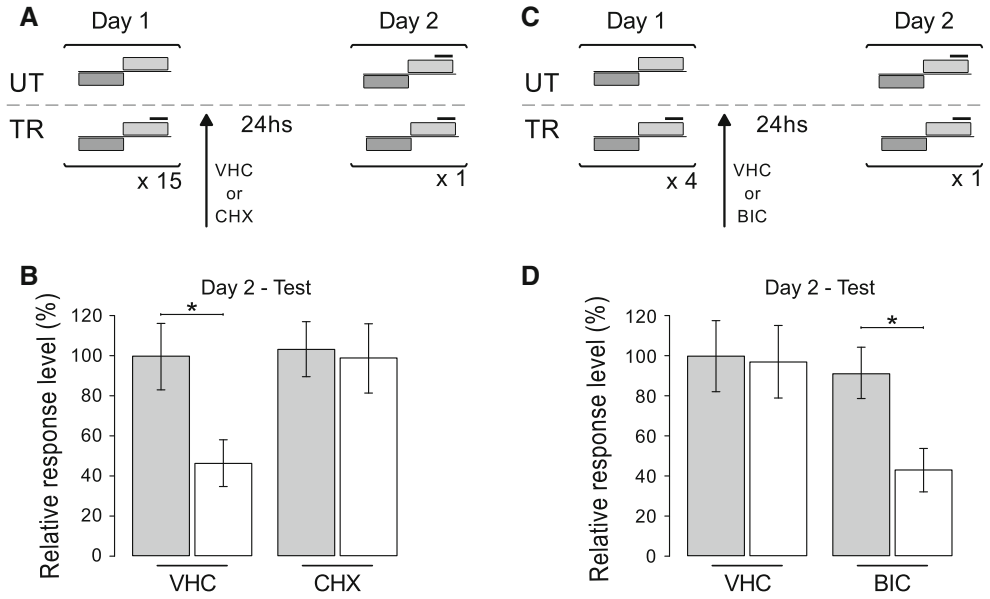

Fig. 6 Experiment 6 and 7. **a** CPC consolidation depends on the synthesis of macromolecules. Experimental protocol. Day 1: two pairs of UT–TR groups were immediately injected after the training session. One pair was injected with VHC and the other with CHX. Day 2: one testing trail. **b** Testing session $*P < 0.05$. **c** CPC consolidation is enhanced by bicuculline administration. Experimental protocol. Day 1: two pairs of UT–TR groups received four training trails (weak training). Immediately after training, one pair of UT–TR groups was injected with VHC and the other pair with BIC. Day 2: one testing trail. **d** Testing session. *Grey bars* stand for UT groups and white for TR groups. $*P < 0.05$

pair received a vehicle injection (VHC). The injections occurred after a weak training protocol that consisted of four trials. One day after training, the animals were tested in one trial (Fig. 6c). Results at testing are presented as in Experiment 1.

Results and discussion

No differences were found between groups for the vehicle pair, as was expected considering the weak training protocol that was provided. However, significant differences between groups were found for the pair that were injected with BIC, which indicates that memory was enhanced during consolidation (Fig. 6d) ($F_{3,116} = 2.96$; $P < 0.05$; UT-VHC vs. TR-VHC: $P = 0.7$ and UT-BIC vs. TR-BIC: $P < 0.05$).

Experiment 8: Pavlovian conditioning undergoes memory reconsolidation

As we mentioned earlier, if a consolidated memory is reactivated, it can become transiently labile again and momentarily sensitive to disruption. After labilisation, a process termed reconsolidation is necessary to maintain the memory (Nader et al. 2000; Anokhin et al. 2002; Pedreira et al. 2002; Eisenberg et al. 2003; Sangha et al. 2003). For contextual memories, reconsolidation is induced by a brief re-exposure to the training context (Pedreira et al. 2002; Suzuki et al. 2004; de la Fuente et al. 2011). Previous work on *Chasmagnathus* has also shown that context re-exposure without reinforcement is a necessary condition for labilisation and reconsolidation induction (Pedreira et al. 2004). NF-kB is a transcription factor that plays a key role in memory consolidation and reconsolidation (Romano et al. 2006b). Sulfasalazine is a specific NF-kB inhibitor that induces amnesic effects in *Chasmagnathus* for both consolidation (Merlo et al. 2002) and reconsolidation (Merlo et al. 2005). In the current study, we used sulfasalazine to evaluate memory disruption during reconsolidation one day after CPC training.

Methods

One UT–TR pair was injected with 5 mM sulfasalazine (SSZ), and the other UT–TR pair was injected with the vehicle (VHC). Twenty minutes after the injection, all of the crabs received one trial of light shift without the visual danger stimulus presentation, which included 27 s of re-exposure to the above-light context (5 min of total re-exposure to the experimental device). Twenty-four hours after the re-exposure session, the crabs received one testing trial with the aversive stimulus presentation (Fig. 7a, upper panel). Then, to evaluate whether the disrupting effect of sulfasalazine was specific to memory reactivation due to context re-exposure, we performed the following experiment using the same groups as in the previous experiment, but only the below-light context was presented during the

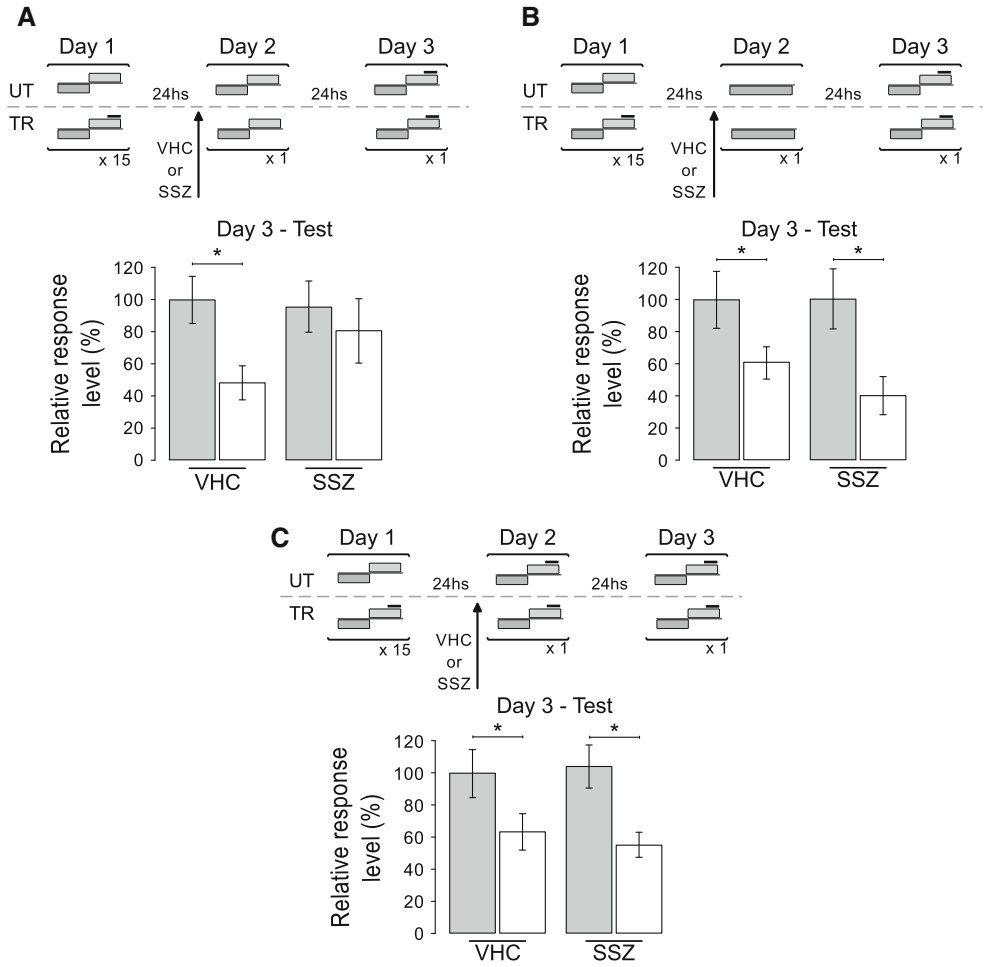

Fig. 7 Experiment 8. **a** *Upper panel* experimental protocol. Day 1: two pairs of UT–TR groups received 15 training trials. Day 2: one pair of UT–TR groups was injected with VHC and the other with SSZ, and 20 min later, both pairs were re-exposed to the training context for 27 s. Day 3: one testing trail. *Lower panel* Testing session. **b** *Upper panel* experimental protocol. Day 1: two pairs of UT–TR groups completed 15 training trials. Day 2: one pair of UT–TR groups was injected with VHC and the other with SSZ, and 20 min later, both pairs of groups were re-exposed to the training context without a light shift. Day 3: one testing trail. *Lower panel* Testing session. **c** *Upper panel* experimental protocol. Day 1: two pairs of UT–TR groups underwent 15 training trials. Day 2: one pair of UT–TR groups was injected with VHC and the other with SSZ. Twenty minutes later, both pairs were re-exposed to the training context, and a US was presented to all groups. Day 3, one testing trial. *Lower panel* Testing session. *Grey bars* stand for UT groups and *white* for TR groups. *$P < 0.05$

re-exposure session. Twenty-four hours after the re-exposure session, the animals were tested in one trial that included the visual danger stimulus presentation (Fig. 7b, upper panel). Finally, we explored the condition that context re-exposure without reinforcement is a necessary condition for labilisation and reconsolidation induction. Thus, in the subsequent experiment, a complete CPC trial, including the visual danger stimulus presentation, was used as a re-exposure session one day after training (Fig. 7c, upper panel). Results at testing are presented as in Experiment 1.

Results and discussion

The UT–TR vehicle pair showed significant differences between groups, but the UT–TR sulfasalazine pair did not (Fig. 7a, lower panel) ($F_{3,116} = 2.73$; $P < 0.05$; UT-VHC vs. TR-VHC: $P < 0.05$ and UT-SSZ vs. TR-SSZ: $P = 0.99$). These results indicated that sulfasalazine administration impaired retention. When we evaluated the effect of the drug without reactivation, in the test session, both the vehicle and sulfasalazine pairs showed significant differences between group (Fig. 7b, lower panel) ($F_{3,116} = 7.79$; $P < 0.05$; UT-VHC vs. TR-VHC: $P < 0.05$ and UT-SSZ vs. TR-SSZ: $P < 0.05$), which indicated that the amnesic effect of sulfasalazine occurred only when the crabs were re-exposed to the above-light context. These findings strongly suggest that the drug acts on reconsolidation and that memory reactivation and labilisation are initiated specifically by re-exposure to the above-light context.

Finally, for the last experiment results when the US was presented with the CS in the reactivation session, the testing trial showed significant differences between groups for

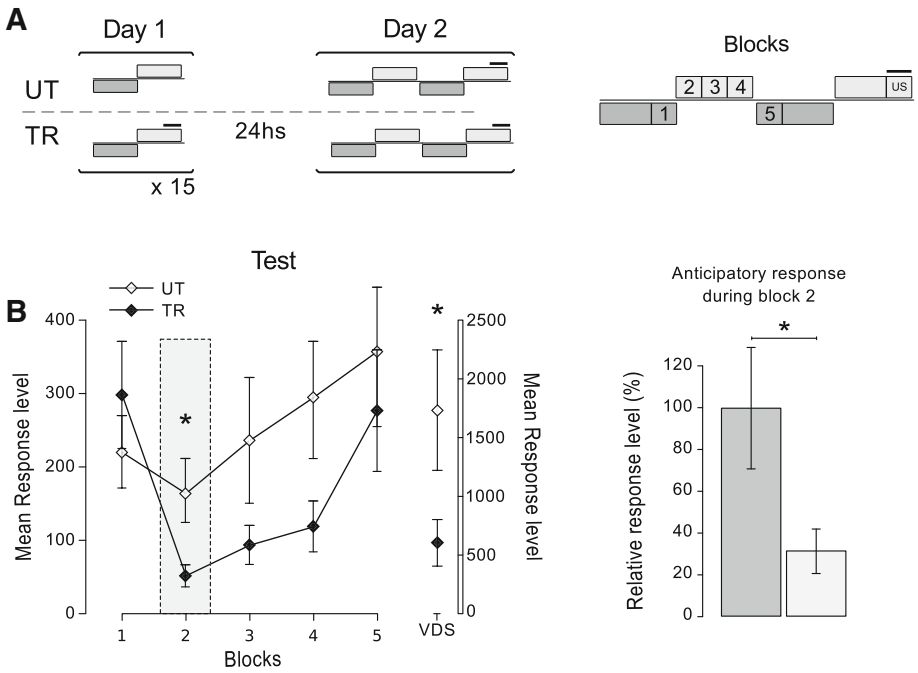

Fig. 8 Experiment 9. **a** *Left panel* experimental protocol. Day 1: training session. One pair of UT–TR groups received 15 training trials. Day 2: testing session. Two testing trials were conducted; during the first, no US was presented to measure the anticipatory response, and in the second, one US trial was presented. *Right panel* blocks of data recorded during the two Day 2 test trials (b1–b5 and US trial). **b** *Left panel* mean responses for the Day 2 test trials. The levels of response during the different recording blocks are shown, as well as the response to the US presentation. *Right panel* mean responses ± S.E.M. during b2, normalised with respect to the mean response of the UT group. *Grey bars* represent the UT groups, and *white bars* represent the TR groups. $*P < 0.05$

both sulfasalazine and vehicle pairs (Fig. 7c, lower panel), which indicated that the reinforced trial was unable to induce labilisation and reconsolidation, impeding the effect of sulfasalazine ($F_{3,116} = 3.69$; $P < 0.05$; UT-VHC vs. TR-VHC: $P < 0.05$ and UT-SSZ vs. TR-SSZ: $P < 0.05$). These findings stress the specificity of this drug, which prevents memory reconsolidation after memory retrieval only for those circumstances in which memory labilisation and reconsolidation have already taken place.

Experiment 9: Pavlovian conditioning induces anticipatory response

Once Pavlovian conditioning has been established, the CS presentation induces an anticipatory response as a predictor of the US occurrence. In contextual conditioning, the CR is expected when animals are re-exposed to the training context. Based on the seemingly stronger contingency established using the CPC paradigm compared with the traditional paradigm, the second series of experiments was aimed at analysing whether an anticipatory response was expressed when the animals were confronted with the training context. Thus, in the following experiment, we explored the occurrence of a freezing response in the presence of the above-light context.

Methods

For this purpose, a UT–TR pair of groups received a training session of 15 trials. One day later, the groups were tested in two trials of light shift. The first trial was presented without the visual danger stimulus, and the second trial was presented with the aversive stimulus (Fig. 8a, left diagram). The first trial was divided into blocks and then analysed. The first group was five blocks (b1–b5) of 9 s each, followed by one block that comprised the final 9 s of the first below-light illumination, three 9-s blocks that comprised the entire period of above-light illumination and one last block that comprised the first 9 s of the inter-trial interval (second light below). The second trial was analysed during the 9 s of the visual danger stimulus presentation (Fig. 8a, right diagram). Results at testing are presented as in Experiment 1.

Results and discussion

During the first block (b1) of below-light exposure, no differences were found between the UT and TR groups (Fig. 8b) ($F_{1,58} = 0.4$; $P = 0.5$). However, after the light shift, the activity of the TR group dropped markedly to low levels, indicating that most of the animals froze (b2) ($F_{1,58} = 5.54$; $P < 0.05$). This difference in activity sup-

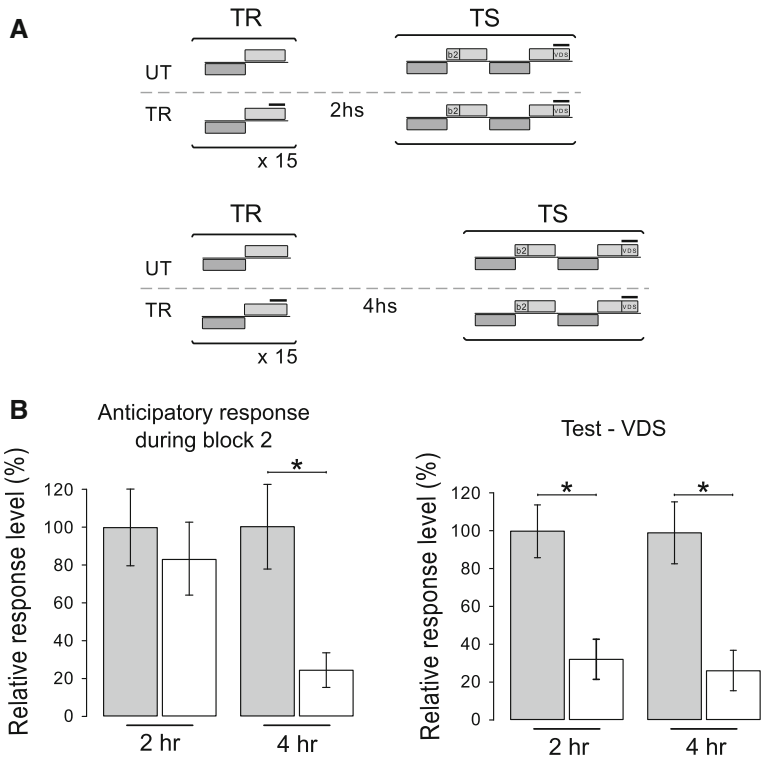

Fig. 9 Experiment 10. **a** Experimental protocol. Two pairs of UT–TR groups received 15 training trails. The testing session was conducted two (*upper panel*) or four (*lower panel*) hours later. **b** *Left panel* mean responses ± S.E.M. during b2 normalised with respect to the mean response of the UT-2 h group. *Right panel* mean responses ± S.E.M. during US presentation, normalised with respect to the mean response of the UT-2 h group. *Grey bars* represent the UT groups, and white bars represent the TR groups. *$P < 0.05$

ports an anticipatory response to the CS (above-light context). During the inter-trial interval with the below-light context, the first 9-s block (b5) again showed the same level of activity for both groups ($F_{1,58} = 1.72$; $P = 0.19$). Finally, in the second trial, the escape response to the visual danger stimulus was measured, and the expected significant differences were observed (Fig. 8b) ($F_{1,58} = 8.34$; $P < 0.05$). Considering that b2 included the light switch, this block appeared to be the best indicator of the anticipatory response. Consequently, we used this block as a genuine index for the anticipatory response (Fig. 8b, b2).

Experiment 10: Short-term memory for anticipatory response is expressed at periods longer than 2 h

In previous studies that used the standard protocol, short-term retention of the response to the visual danger stimulus presentation was attributed to a non-associative memory component. Such a process induces a general reduction in the response that is not specific to the stimulus or the context characteristics (Romano et al. 1991). The following experiment was aimed at evaluating the presence of short-term memory (STM) that was induced by the CPC training protocol and to assess the possible differences between the expression of anticipatory freezing as associative memory and the expression of freezing in response to the US as non-associative memory.

Methods

Two pairs of UT–TR groups were trained for 15 trials and were tested in two trials at either 2 or 4 h after training. The first trial consisted of a light shift without presentation of the visual danger stimulus, and the second consisted of a light shift with the aversive stimulus presentation (Fig. 9a). Results at testing are presented as in Experiment 1.

Results and discussion

b2 from the first trial showed significant differences at 4 h but not at 2 h (Fig. 9b) ($F_{3,116} = 3.24$; $P < 0.05$; 2 h $P = 0.25$ and 4 h $P < 0.05$). The second trial showed significant differences between the UT and TR groups at both 2 and 4 h ($F_{3,116} = 5.65$; $P < 0.05$; 2 h $P < 0.05$ and 4 h $P < 0.05$). We concluded that at 2 h, the associative memory was not completely consolidated and thus not expressed. The freezing in response to the US that was observed at 2 h was attributable to the non-associative component. Conversely, at 4 h after training, both memory components were present and expressed.

General discussion

In the present work, we obtained evidence that indicates that the crab *Chasmagnathus* establishes an association between a particular context and an aversive stimulus,

termed the visual danger stimulus (unconditioned stimulus, US). This association showed the characteristics of Pavlovian conditioning. We initially found that this association occurred only when it was presented in a paired manner. A strictly non-paired design rendered no freezing in response to the US, indicating that the aversive stimulus was not associated with the general features of the experimental device but with the particular traits of the above-light context. This result allows us to rule out the interpretation that the learning was a case of habituation because the animals that were trained with the non-paired design showed no retention, although they received a strong training with the US presentation. However, it is also possible that a high level of response occurred at testing when the US was more salient for the trained animals in the non-paired condition because the TR non-paired group received the same stimuli under two different salience conditions. Additionally, another associative phenomenon may be considered for the retraining phase. Both UT groups were exposed to 15 illumination changes without the US. Thus, it is also possible that both UT groups showed a latent inhibition during the testing session because they were more resistant to acquiring the freezing response. Consequently, a significant difference was found in the UT groups compared with their respective TR groups due to this associative process.

As occurs in Pavlovian conditioning, the simultaneous onset design in which the US was presented at the same time as the above-light context caused no retention in the first testing trial. Conversely, a significant reduction in the response to this context was found in trials 2–6. These findings indicate that under the simultaneous onset conditions, no association between the context and the US was established, but the repeated presentation of the US induced a habituation that was expressed during the retraining phase of testing. A difference between the pairs would be predicted if the TR-F group acquired the conditioned response during training while the TR-S failed to acquire the response because of the temporal stimuli arrangement. However, the absence of this difference between the types of training may be explained by the independence of the intrasession diminution of the response level from the contextual cues and its exclusive dependence on the aversive stimulus (Suárez et al. 2010). Further, we found that the freezing response that was acquired during training was not displayed when the relevant contextual visual characteristics were changed between training and testing (striped container vs. blank container). In such conditions, retention was not present despite identical illumination in both contexts. These new results support previous research that has demonstrated that crabs perceive the same context under different illumination as different environments (Pérez-Cuesta and Maldonado 2009; Hepp et al. 2010). This difference in perception indicates that the above-light illumination did not act as a cue that was associated with the US by itself. This result could be considered unexpected because the above light had a separate onset in each trial and, during training, gained a high predictive value for the US because it preceded the presentation of the US. However, these characteristics were not sufficient to transform the light into a cue stimulus on its own, and it was only in combination with the context traits that the light became the CS in this new paradigm. The above light may also represent an occasion setter that allowed the animals to perform the conditioned response when this complex CS was presented to them (Holland 1983; Brembs and Wiener 2006).

In the traditional training procedure, the above-light context was present during the entire training session. Under these conditions, we were unable to find a measurable anticipatory conditioned response when the animals were re-exposed to the training context at testing because of the light's poor value as a predictor of the US onset. Conversely, with the new procedure, we found a clear anticipatory response after the illumination was shifted from below to above during testing. Under this protocol, the trained animals displayed freezing and thus significantly reduced their level of spontaneous activity. This salient difference in behaviour may have been a consequence of the strong contingency that was established between the context illuminated from above and the US. Thus, the new protocol was found not only to establish an association with the context (the blank container illuminated from above), as in the traditional protocol, but also to build a strong relationship between both stimuli (context and US) that was reflected in both the established contingency (Fig. 3) and the temporal relationship required for the stimuli to develop the conditioning (Fig. 4).

During training, the crabs were exposed to two contextual conditions, but only one condition was presented simultaneously with the US. We have interpreted such design differences as differential conditioning. The above-light context can be considered the CS+, and the below-light context can be considered the CS−. The lack of an anticipatory response during the below-light presentations (blocks 1 in Fig. 8b) supports such an interpretation.

We have interpreted the present memory paradigm as contextual conditioning. However, the phasic presentation of the context could be understood as a cued conditioning, in which the US is signalled by some traits of the above-light context. We consider such an interpretation to be less than tenable. The light above the container was not assigned as a cue because despite its recognisability as a US predictor due to its discrete onset, the light also produced enough diffuse illumination to affect the context details. Similarly, the remaining elements in the experimental device were too complex to be interpreted as cues, and none of these elements were individually salient. Evidence

for this conclusion emerged from the analysis of the context-shift experiment (Fig. 4). In this experiment, the animals were subjected to changes in illumination during training, but the other visual cues were quite different (i.e. black–white bars). Therefore, despite the illumination changes in both sessions, the changes in the other cues hindered the expression of the LTM that was acquired under these special circumstances. In this condition, the above light could have acted as a cue similar to an occasion setter (Holland 1983; Brembs and Wiener 2006). In this experiment, the context cues as a whole, including the above light, acted as the CS. Conversely, a spot light can be used as the CS to induce autonomic response conditioning in these crabs (Burnovicz and Hermitte 2010).

In the current study, we found memory features in the CPC that were common with other memories for associative learning at both the behavioural and mechanistic levels. Initially, an STM was expressed 2 h after training. Notably, this memory was not expressed under the presentation of the CS, but only under the presentation of the US as freezing in response to the US. Conversely, 4 h after training, freezing was displayed under the presentation of both the CS and US. We postulated that initially, a non-associative STM was expressed, and then, once the consolidation of this memory was complete, the STM was replaced by the associative LTM. Further experiments are needed to evaluate whether the memory that was expressed 4 h after training contains the same characteristics as the memory that was present one day after training. Previous work with this model indicates that short-term retention is expressed by an unspecific opioid-dependent mechanism (Romano et al. 1990).

A universal characteristic of LTM consolidation is its dependence on protein synthesis during the first few hours after training. As expected, the consolidation of CPC memory was sensitive to cycloheximide when the drug was administered immediately post-training. Furthermore, during consolidation, the CPC memory was enhanced by means of bicuculline administration. This antagonist has previously been shown to have facilitatory effects on memory in different species, including the crab *Chasmagnathus* (Carbó Tano et al. 2009).

As with other associative memories, we found that CPC memory underwent extinction when the crabs were exposed to the above-light context one day after training. Extinction was achieved only when the animals were exposed to the context for as long as 2 h, similarly to the traditional training without a light shift (Pedreira et al. 2004; Hepp et al. 2010). Moreover, the process was not triggered when the animals were exposed to 15 or 30 changes in the illumination, but when the amount of time spent in the context was less than 2 h (data not shown). As a whole, these results support the idea that the contextual cues act as the real CS. We found that CPC memory underwent reconsolidation as well. Labilisation–reconsolidation was achieved 1 day after training when the crabs were re-exposed to the above-light context without reinforcement for only 27 s. The induction of such a process was made evident by the administration of sulfasalazine, which is commonly used as an amnesic agent in memory studies of crabs and rodents (Merlo et al. 2002; Freudenthal et al. 2005; Boccia et al. 2007; Lubin and Sweatt 2007). This drug interferes with the activation of NF-kB, which is a key transcription factor for memory re-stabilisation after labilisation induction (Merlo et al. 2005; Boccia et al. 2007; Lubin and Sweatt 2007; de la Fuente et al. 2011).

Both the CS and US used in the current study are, in principle, stimuli of the same sensory modality. The overall context is potentially processed from multi-sensorial information; however, visual information appears to be the key source of information that was processed by the crabs in these experimental conditions. In this same laboratory, we have changed different characteristics of the context, such as the presence of odorants, tactile stimulation and the presence or absence of water (unpublished results), but context-specific characteristics were found only in the visual modality. The US that we used here is entirely visual, and an important body of information has been obtained in the last decade regarding the neural substrates of the visual danger stimulus detection and the neurophysiological processing that is involved in learning. Lateral giants are movement detector neurons that are present in the third visual neuropil of the crab, which is termed the medulla (Berón de Astrada et al. 2001). The activity of these neurons is tightly correlated with crab behaviour, and they are considered to play a key role in the determination of the US-elicited response and in the decay of the response, both during the training and in testing. Such a correlation was found for both massed and spaced training (Tomsic et al. 2009). However, under contextual changes, these animals showed high levels of response, while the LG neurons continued to respond at low levels to the US (Sztarker and Tomsic 2011). These findings indicate that (a) the contextual information is processed in other neuronal circuits and (b) the integration of both the US and contextual information and the subsequent behavioural outcome occur downstream from the LG circuits.

The new paradigm developed in the present paper may be a powerful tool in the search for the neuronal substrates of context representation due to the possibility of phasic presentations of the context (Sztarker and Tomsic 2011). Moreover, the adjustment of this new paradigm to the definition of Pavlovian conditioning allows comparative studies with other paradigms that may reveal the shared components that exist at a molecular level.

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Ethical standards Experimental procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (USA), and the Argentinean guidelines on the ethical use of animals. All the experiments performed in this work were planned minimising the number of animals used and their suffering.

Conflict of interest The authors declare that they have no conflict of interest.

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