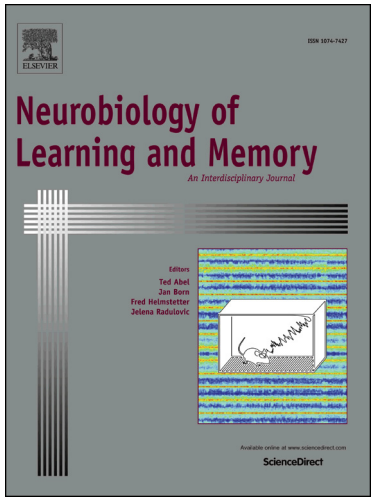


Neural correlates of expression-independent memories in the crab *Neohelice*

F.J. Maza, F.F. Locatelli, A. Delorenzi

PII: S1074-7427(16)30001-6
DOI: http://dx.doi.org/10.1016/j.nlm.2016.03.011
Reference: YNLME 6413

To appear in: *Neurobiology of Learning and Memory*

Received Date: 6 February 2016
Revised Date: 9 March 2016
Accepted Date: 12 March 2016

Please cite this article as: Maza, F.J., Locatelli, F.F., Delorenzi, A., Neural correlates of expression-independent memories in the crab *Neohelice*, *Neurobiology of Learning and Memory* (2016), doi: http://dx.doi.org/10.1016/j.nlm.2016.03.011

Title:

Neural correlates of expression-independent memories in the crab *Neohelice*.

Running title: Neural correlates of expression-independent memory.

Keywords: memory, reconsolidation, retrieval, memory expression, calcium imaging

Pages: 46

Figures: 5

Tables: 1

Word Counts (total): 14081

Word Counts (abstract): 213

Neurobiology of Learning and Memory, Editorial Office

Article Type: Research Reports

Authors: Maza F.J.; Locatelli, F.F. ; Delorenzi A.

-Corresponding Author: Alejandro Delorenzi. delorenzi@fbmc.fcen.uba.ar -Phone: 54-11-4576-3348 - Fax: 54-11-4576-3447

Institution: Laboratorio de Neurobiología de la Memoria, Departamento de Fisiología y Biología Molecular, IFIByNE-CONICET, Pabellón II, FCEyN, Universidad de Buenos Aires, Ciudad Universitaria (C1428EHA), Argentina. Phone: 54-11-4576-3348 - Fax: 54-11-4576-3447.

-Francisco Javier Maza. fjmaza@fbmc.fcen.uba.ar

-Fernando F. Locatelli. locatellif@fbmc.fcen.uba.ar

-Alejandro Delorenzi. delorenzi@fbmc.fcen.uba.ar

Abstract

The neural correlates of memory have been usually examined considering that memory retrieval and memory expression are interchangeable concepts. However, our studies in the crab *Neohelice* (*Chasmagnathus*) *granulata* and in other memory models have shown that memory expression is not necessary for memory to be re-activated and become labile. In order to examine putative neural correlates of memory in the crab *Neohelice,* we contrast changes induced by training in both animal´s behavior and neuronal responses in the *medulla terminalis* using in-vivo Ca2+ imaging. Disruption of long-term memory by the amnesic agents MK-801 and scopolamine (5µg/g) blocked the learning-induced changes in the Ca2+ responses in the *medulla terminalis*. Conversely, treatments that lead to an unexpressed but persistent memory (weak training protocol or scopolamine 0.1 µg/g) do not block these learning-induced neural changes. The present results reveal a set of changes in the neural activity induced by training that correlates with memory persistence but not with the probability of this memory to be expressed in the long-term. In addition, the study constitutes the first in-vivo evidence in favor of a role of the *medulla terminalis* in learning and memory in crustaceans, and provides a physiological evidence indicating that memory persistence and the probability of memory to be expressed might involve separate components of memory traces.

1. Introduction

Current hypotheses for the study of the neurobiological basis of learning and memory suggest that experience is encoded and retained by the nervous system as changes in synaptic efficacy and neuronal restructuration (Dudai, 2002a; b). The changes that encode a memory are complex to delineate since they might be disseminated across several brain areas. Establishing the link between specific neuronal changes and specific memory features is essential to understand how the memory is built (Davis, 2011; Dudai and Morris, 2013; Lukowiak, Sangha, Scheibenstock, Parvez, McComb, Rosenegger, Varshney, and Sadamoto, 2003; Menzel, 2014; Morris, 2007). However, since the existence of the memory trace and its many building blocks are accessible to the observer only through memory expression, a difficulty in that search is that a lack of change at the behavior might be misinterpreted as a lack of memory. In this regard, reconsolidation experiments are helpful to bring to light memories that are consolidated but remain behaviorally unexpressed (Delorenzi, Maza, Suarez, Barreiro, Molina, and Stehberg, 2014; Dudai and Morris, 2013). For instance, reconsolidation experiments in the crab *Neohelice granulata* has allowed us to reveal memory persistence independently of memory expression (Barreiro, Suarez, Lynch, Molina, and Delorenzi, 2013; Caffaro, Suarez, Blake, and Delorenzi, 2012; Frenkel, Maldonado, and Delorenzi, 2005b; Frenkel, Suarez, Maldonado, and Delorenzi, 2010b).

In the *Neohelice* context-signal memory (CSM) paradigm, crabs associate the training context with a visual danger stimulus (VDS) passing overhead. After the repeated presentations of the VDS, the escape response is replaced by a freezing response to the VDS. Several neuronal correlates of the CSM have been studied in the central brain and optic lobes using biochemical, molecular and electrophysiological approaches (Tomsic and Romano, 2013). A group of motion sensitive neurons that projects from the lobula to the lateral protocerebrum reflects some short-

and long-term behavioral changes induced by CSM training (Sztarker and Tomsic, 2011; Tomsic and Romano, 2013). Here, we focus on the *medulla terminalis* that form part of the lateral protocerebrum and is proposed to function as a region of high-order multimodal integration that process at least chemical, visual and mechanical information (Blaustein, Derby, Simmons, and Beall, 1988; Sullivan and Beltz, 2001).

In the present study, we combine behavioral and neuronal recordings in the crab memory model to examine putative neuronal correlates of memory. Using in vivo calcium imaging, we recorded neural activity elicited by the training stimulus in the *medulla terminalis* and evaluated learning induced changes. By contrasting how behavioral memory and changes in neuronal activity in the *medulla terminalis* are altered by different training protocols and by the amnesic agents scopolamine and MK-801 (Beron de Astrada and Maldonado, 1999; Caffaro et al., 2012; Troncoso and Maldonado, 2002), we conclude that the changes in the medulla terminalis reflect the formation of a persistent memory trace, independently of its probability to be behaviorally expressed in the long term.

2. Materials and Methods

2.1 Animals

Intermolt adult male crabs of the species *Neohelice (Chasmagnathus) granulata* measuring between 2.7- 3.0 cm across the carapace were collected from the narrow coastal inlets of San Clemente del Tuyú, Buenos Aires Province, Argentina. In the laboratory, crabs were kept on a 12:12 hours light-dark cycle, in collective plastic tanks (20 animals each) filled up to 2 cm depth with 12% seawater prepared with hw-Marinex (Winex, Germany) salt, pH 7.4-7.6. The holding and

experimental rooms were kept at 22-24ºC and 80 ± 10% relative humidity. Experiments were carried out at daytime within fifteen days after the animals have been captured. Each crab was used only in one experiment. All efforts were made to minimize the number of animals. All the research was conducted in accordance with the Ethical Reference Frame for Biomedical Investigations of the CONICET, equivalent to the standard procedures for animal care and use of the NIH of the U.S.A.

2.2 Behavioral procedures

Context Signal Memory (CSM)

The experimental device, the actometer (Maldonado, 2002; Tomsic and Romano, 2013), referred to as the training context (TC), consisted of a bowl-shaped opaque container with a steep concave wall 12 cm high (23 cm top diameter and 9 cm floor diameter) covered to a depth of 0.5 cm with fresh 12% artificial seawater, where the crab was lodged during the training and the testing sessions of the behavioral experiments. During each trial, an opaque rectangular screen (25 x 7.5 cm), termed VDS for visual danger stimulus, was moved twice horizontally over the actometer at a constant speed. The movement of the VDS was driven by a motor at an angular velocity of 82°/sec. The motion cycle, which consisted of a 90° clockwise and counterclockwise excursion from the starting position and back, was completed in circa 2.2 sec. Each recording trial was composed of two cycles separated by 2 sec. The trial was built in this way to make certain that the passing screen enters the animal´s visual field twice during each trial, thus ensuring animals are similarly stimulated regardless of their positions inside the recording container. The VDS presentation induces an escape response in the crabs and consequent container vibrations that are converted into electrical signals through four microphones placed on the external wall of the container. The activity of the crab was recorded throughout each trial

in arbitrary units. The experimental room has 40 actometers, separated from each other by partitions, and animals from different experimental groups can be treated simultaneously. During the CSM experiments, crabs were illuminated with a 5 W light bulb, from above the training context. A Strong Training Protocol consisted of fifteen VDS trials separated by 3 minutes intervals. The expression of CSM is revealed at testing session as a significant decrease in the escape response of the animals upon stimulation with the VDS. CSM can be revealed for at least 5 days (Maldonado, 2002; Romano, Locatelli, Freudenthal, Merlo, Feld, Ariel, Lemos, Federman, and Fustinana, 2006; Tomsic, de Astrada, Sztarker, and Maldonado, 2009; Tomsic, Pedreira, Romano, Hermitte, and Maldonado, 1998). After a weak training protocol, consisting of six spaced trials, crabs show memory expression when tested in the short term (up to 4 h after weak training) but not in the long term (Smal, Suarez, and Delorenzi, 2011). In experiments in which short-term memory was tested (here 30 minutes after training), the illumination of the training context was turned off one minute after the last training trial and turned on again 25 minutes later. The change in illumination is equivalent to removing the animal from the training context (Perez-Cuesta and Maldonado, 2009). The short-term test, consisted of one VDS trial, carried out 5 minutes after the light was turned on.

The reconsolidation hypothesis proposes that a consolidated memory enters an unstable state when re-activated (memory labilization), becoming transiently sensitive to disruption or enhancement by several agents (Lewis, 1979; Pedreira and Romano, 2013). Therefore, for in reconsolidation experiments, three sessions were included: Training Session (Day 1), Reminder Session (Day 2) 24 h after training, and Testing Session (Day 3) 48 h after training.

During the experimental protocols, crabs were regularly distributed into pairs of groups, each pair consisting of a trained group (TR) and an untrained (control) group (UN). Untrained

animals remained in the actometer during the entire Training Session, but received only one training trial with the VDS that was simultaneous to the first training trial of the trained group. This trial was used to control homogeneity in the escape response of all experimental groups at the beginning of the experiments. In the Reminder and Testing Sessions, UN groups were treated equal as TR groups. Each group had between 25 and 40 crabs.

Day 1, Training session. TR group: This group underwent a training protocol. First, each crab was placed in the training context (the actometer) for 10 min without being stimulated with the VDS. After this adaptation period, animals received 15 training trials (or 6 in case of weak training protocol), separated by inter-trial intervals of 3 min. UN group: Each crab of the untrained group was placed in the training context and remained there the same time as the trained animals. Untrained animals were stimulated with the VDS only in the first trial. When the session was finished, TR and UN animals were immediately placed in individual resting containers until the following session.

Day 2 – Reminder session. The animals were subjected to one of two different treatments during this session (Maldonado, 2002; Pedreira et al., 2004):

(a) Crabs were re-exposed to the training context (TC) for 5 min without the VDS presentation. This procedure reactivates memory and turns it into a labile state initiating a reconsolidation process.

(b) Crabs were re-exposed to the training context (TC) for 5 min, and the VDS was presented once at the end of the exposure (TC+VDS). The culmination of the reminder session with the presentation of the VDS prevents memory labilization because reconsolidation is not initiated.

After the presentation of the incomplete (a) or complete (b) reminder, the crabs were returned to the resting containers and were water deprived for 2 h in order to enhance memory expression. It has been previously shown that water deprivation during reconsolidation enhances memory expression after a weak-training protocol and also after saralasin or scopolamine-induced amnesia (Caffaro et al., 2012; Frenkel, Freudenthal, Romano, Nahmod, Maldonado, and Delorenzi, 2002; Frenkel, Maldonado, and Delorenzi, 2005a; Frenkel et al., 2005b; Frenkel et al., 2010b).

Day 3 – Testing session. On the third day, all crabs were reinstalled in the training context for 10 min and memory expression was tested with a VDS presentation.

2.3. Optical imaging experiments

2.3.1. Crab preparation and staining

The crabs were anesthetized in ice-cold water for preparation. During preparation and measurements, the chelae were immobilized with a rubber band and the crabs were firmly held by their exoskeleton with an adjustable clamp that allowed free movements of the legs (Figure 2A). The right eyestalk was covered with wet paper in order to avoid visual stimulation during the preparation. The left eyestalk was fixed with instant acrylic glue (*La Gotita*, Akapol S.A.)(Figure 2B). A small container was build with dental cement (Dycal, Milford, USA) around the eyestalk to allow continuous flow of crab Ringer´s solution (NaCl, 468.00 mM; KCl, 9.46 mM; MgCl2, 7.50 mM; CaCl2, 12.53 mM; Hepes, 5.00 mM; NaHCO, 37.00 mM; Glucose, 2 mM; Osmolarity, 1045.00 mOsm (Genovese, Senek, Ortiz, Regueira, Towle, Tresguerres, and Luquet, 2006)). Then, a window (circa 3 x 2 mm) was opened in the cuticle of the stalk using a sharp scalpel. To allow visual access to the *lateral protocerebrum*, the connective tissue and muscles were removed, the neuroepithelium was cut along the longest axis of the eyestalk with a vannas

scissor (WPI 555640S, Sarasota, FL) and attached to lateral and medial edges of the window (Figure 2C). Borosilicate glass electrodes (outer diameter, 1.2 mm; inner diameter, 0.69 mm; length 100mm; *Sutter Instruments*) were pulled with a horizontal puller (P97; *Sutter Instruments*). A glass microelectrode was coated with dye crystals (Calcium Green™-1 dextran, Potassium Salt 3000 MW Anionic, *Molecular Probes®, Life Technologies*) and stabbed into the *lateral protocerebrum* where multiple neuronal processes of the *medulla terminalis* can be reached (Figure 2D-E)(Galizia and Vetter, 2004). Successive washes with Ringer´s solution were carried out to clean the dye that remained outside the tissue. The approximate area of optical imaging of the stained neuronal process by backfilling is indicated in Figure 2D; somata were rarely observed in the recorded area. After calcium imaging, optic ganglia were dissected and fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). Tissues were dehydrated in increasing ethanol concentrations in PBS and mounted in methyl salicylate (Sigma-Aldrich, St. Louis, MO, USA)(Frenkel, Dimant, Portiansky, Imboden, Maldonado, and Delorenzi, 2010a). Fluorescence in fixed tissue samples was visualized using a confocal microscope (Olympus FV300, Japan) with a 488-nm argon laser, an UplanFl 20× objective (NA 0.5), a dicroic cube SDM 570 to split the acquisition channel and an emission BA510 (Olympus Optical). Images´s pixel size was 0.69 µm/pixel. Data was recorded by using FluoView Software and saved in a 12-bit TIFF format. Frame brightness and contrast were adjusted by using ImageJ software (ImageJ1.45s Wayne Rasband, National Institutes of Health, USA) (Schneider, Rasband, and Eliceiri). The ImageJ software maximum projection function was used for stacks. Figure 2E shows an example of a stack of confocal images showing the neuronal processes in the *medulla terminalis* that were stained with Calcium Green-1. Figure 2F shows the hemiellipsoid body neuropil which is located at least 100 µm below the focus plane of the registered *medulla terminalis*, in the opposite side of the lateral protocerebrum (Frenkel et al., 2010a).

2.3.2. Experimental procedures

Optical imaging experiments were performed inside a cage completely covered with black cloth to prevent undesired visual stimulation. The top of the custom-made anti-vibration table was painted black. Illumination within the cage was provided by an LED oriented toward the roof of the cage. The light reached the crab's eye with an intensity of circa 200 mW/m2. Experiments started between 1.5 and 2 h after dye application. Alternations between two different contexts were produced by turning on and off the illumination inside the cage (Perez Cuesta and Maldonado, 2009).

During calcium imaging recording the crabs were held with half of their body submerged in brackish water and the prepared eye under continuous superfusion with Ringer´s solution. Recordings began 15 minutes after the animal has remained visually undisturbed in the recording position. Visual stimulation was carried out through the contralateral eye. In all animals, we stimulated the right eye and recorded signals in the *medulla terminalis* in the left eyestalk. The visual stimulus was analogue of the VDS used in behavioral experiments (size 7.5 - 2.25 cm, distance adjusted to present the same angular size to the animal). As in behavioral experiments, a VDS trial consists of two cycles separated by 2 sec, each one with a 90° clockwise and counterclockwise excursions from the starting position and back completed in 2.2 sec (Figure 4A). Mechanical stimulus (MS) consisted of a pulse of nitrogen gas (10 psi) delivered using a PV830 Pneumatic Pico Pump (WPI Sarasota, FL) by positioning a tip (1mm aperture) at approximately 0.5 centimeters of the right backside of dorsal carapace. Each MS trial consisted of two 1 sec pulses separated by 2 sec (Figure 4B). MS trials were run before training (MS PRE, 1 and 2) and after testing (MS POST). Context illumination, MS and VDS stimulation were controlled from the acquisition software TillVision v4.01 (TILL Photonics Imaging System

Software; TILL Photonics, GmbH). After imaging, animals remained healthy, with no evidence of deleterious effect. During imaging, crabs are half-submerged in brackish water and held in a way that allows free movement of the legs. It is worth to mention that crabs that receive a strong training while being restrained as they were in this study are able to learn and exhibit normal LTM (Tomsic, Maldonado, and Rakitin, 1991; Tomsic and Romano, 2013).

2.3.3. Acquisition and data analysis of Calcium imaging

Optical recordings were carried out using a Nikon E-600 microscope (Nikon, Japan), a 20X, NA=0.95 water immersion objective (Olympus) and a calcium imaging system (Till-Photonics, Germany). Images were taken under 499 nm excitation light at a rate of 10 images per second (10 Hz). Imaging measurements lasted 20 sec (200 frames) and stimuli started at the beginning of the fourth second. Exposure times varied between 15 and 60ms across preparations depending on the intensity of staining and aiming at getting signals within the linear range of the imaging system. Dichroic mirror was 525 nm and emission filter 530 nm LP. Images were taken with a CCD camera (12 bits, 640 x 480 pixels, Imago, Till-Photonics, Germany). Imaged were binned on chip to 320 X 240 pixels, resulting in a pixel size of 1.46 x 1.46 μm. Data acquisition was done using TillvisION v4.01 software. Although the statistical analysis involved trials 1, 6, 15 and test, all trials were recorded. Imaging data analysis was done using custom written macro routines in ImageJ. Raw images were spatially filtered with a median filter (3 x 3) to remove *salt-and-pepper* noise. Displacements between measurements (trials) were corrected before analysis. The analysis of the calcium signals was based on % deltaF/F calculated for each pixel. F represents the basal fluorescence and deltaF the difference between the basal fluorescence and the fluorescence measured during the frame of interest. The basal fluorescence was calculated for each measurement by averaging five frames before stimulus onset. For

visualization of the spatial pattern of the calcium signal, we calculated false-color-coded images that represent the maximum value recorded for each pixel within 3 sec after stimulus onset (for spatial correlation analyses in Supporting Information Figure S1) or the summation of the %deltaF/F along all frames during the stimulus cycle.

An initial analysis of the Ca2+ signals [custom written software by G. Galizia (Konstanz University, Germany) in IDL (RSI, Boulder, Colo., USA)] was performed based on 5 AOIs homogeneously distributed in different areas of the *medulla terminalis* that showed calcium signals elicited by the VDS. Sampling different areas was designed to detect differential changes induced by training. However, the changes turned out to be global increases or decreases in the response to the stimuli without differential contributions of the selected areas. The observation that no area decreased or increased more than another was confirmed by a pixel-wise correlation analysis of the images of activity obtained throughout training and testing trials (Supporting Information, Figure S1). The correlation coefficients between the first and second trial with the VDS were not different from the correlation coefficients between the first and testing trial, and in both cases the coefficients were above 0.8, thus we could not find a significant change caused by training in the spatial pattern of the calcium signal. Because of this, for time-course analysis of the calcium signals the analysis was simplified and performed based on the activity determined in only one AOI per animal that covers all the region of the *medulla terminalis* in which a calcium response was above background activity in any of the recorded trials. The AOI for each crab was semi-automatically created using ImageJ following the next procedure: images representing the average %deltaF/F during the first 2 seconds after stimulus onset were gaussian blurred (sigma=2) and binarized setting a threshold of 5 SDs above baseline. The pixels defined by that threshold constituted the area of activity for a trial. The

biggest area of activity obtained for a crab across all trials was used as mask to define the AOI that was used across all trials. Thus, the change of activity across trials was calculated by averaging the % deltaF/F values of all pixels inside the AOI. Dye bleaching during each measurement was corrected by subtracting a logarithmic curve fitted to the brightness profile. In this way, an average *medulla terminalis* Ca2+ response was obtained for each crab.

2.4. Drug treatments

Crustacean physiological saline solution (Hoeger and Florey, 1989) was used as vehicle. Fifty microliters of vehicle or drug dissolved in vehicle solution were given 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 (Maldonado, 2002). Injections were carried out 15 minutes before starting the corresponding training protocol in both, behavioral and imaging experiments, injecting vehicle solution for every experiment where no drug treatment was used. NMDA receptor ion-channel blocker, MK801 (Tocris Bioscience, Bristol, United Kingdom), and muscarinic cholinergic antagonist, Scopolamine (Sigma Aldrich), were used.

2.5. Statistical analysis

2.5.1. Behavioral experiments, memory retention criterion and data analysis

The escape response values resulting from the 9 s integration that lasted each trial were further transformed by log2 to get normal data distribution. In the experiments in which there where two trained groups, the training session was analyzed using two factors ANOVA: one factor corresponded to the 2 groups of crabs and the 2nd factor corresponded to the 15 trials that were taken as repeated measures. . Mean and Loftus-Masson Confidence Interval (CI 95%) is shown in the figures since this confidence interval provide concordant information with the within

subjects ANOVA error term (Loftus and Masson, 1994). Statistical analysis of CSM expression was assessed by focusing on test trial scores and looking for statistical differences between response levels of the trained (TR) and the untrained (UN) groups within each pair (Maldonado, Romano, and Tomsic, 1997). Rescorla (Rescorla, 1988) convincingly argued in favor of this analysis instead of a within group training vs. testing comparison to clearly distinguish between time of input (training session) and time of assessment (testing session). In the *Neohelice* memory model, a significant difference between the escape response at testing of TR- and UN-groups is invariably observed 24 hours after training (UN>TR), provided that both groups are formed of 25 or more crabs and that the trained group is given 15 or more training trials with a 3-min inter-trial interval. Thus, operationally defined, a TR-group is considered to express the memory when its escape response at the test trial is statistically lower than the escape response of its respective UN-group, which has had exactly the same manipulation and treatment with exception of the training itself. Therefore, the statistical analysis used here, in agreement with all previous studies in this memory model, is based on the prediction that escape response of the UN-group is higher than in the TR-group and thus data are analyzed using a priori LSD planned comparisons (orthogonal contrast analysis, three types of contrasts per experiment) (Howell, 1987; Rosenthal and Rosnow, 1985) following a significant main effect in a one-way ANOVA. In summary, when the experimental design consisted of two pairs of UN-TR groups, the analysis was based on three planned contrasts: a) between the two UN groups, to look for any effect on escape response that might be caused by the differential treatment and not by the training; b) between the UN and TR groups that were subjected to one treatment; and c) between the UN and TR groups that were subjected to a second treatment (eg.(Maldonado, 2002; Pedreira, Dimant, and Maldonado, 1996; Pedreira, Perez-Cuesta, and Maldonado, 2004; Pereyra, Gonzalez, and Maldonado, 2000; Perez-Cuesta and Maldonado,

2009; Romano et al., 2006; Tomsic et al., 1998)). When only one pair of groups (UN and TR) was compared, a t-test was used. For these analyses, mean and standard error of the mean are shown.

2.5.2. Optical imaging data analysis

The calcium imaging data from VDS trials used for statistical analysis corresponds to the summation of positive %deltaF/F values of frames 30-72 (VDS first cycle), and 73-115 (second cycle). For mechanical stimulus trials, the data corresponds to frames 30-40 (MS first pulse), and 63-73 (second pulse) (*Experimental procedures* and Figures 4A, B). Ca2+ signals in response to the VDS were analyzed with 6 x 2 repeated measures ANOVAs using groups and trials as the within subjects factors (Trial 1, TEST), follow-up one-way repeated measures ANOVA were conducted and post-hoc analyses with Fisher's least significant difference were performed to compare between training and testing trials in each group separately. When necessary, Greenhouse-Geisser or Huynth-Feldt correction was used in cases of violation of sphericity. The data shown in the figures correspond to mean ± $CI(95\%)_{Loftus\&Masson}$, since this error is recommended for graphical representations of repeated measures ANOVA (Loftus and Masson, 1994). *t*-test for dependent samples were performed when only two trials were compared. Responses toward MS were compared using a *t*-test for dependent samples, MS PRE (first trial) vs. MS POST. For the comparison of the spatial activity patterns (Figure S1), a Pearson´s R correlation value between false-color pictures was obtained considering the average of areas of 7 x 7 pixels (Approximately 10.2 µm x 10.2 µm) using the plug-in Image CorrelationJ for ImageJ (Chinga and Syverud, 2007). R values between conditions were analyzed by *Sign Tests* for low samples as described in (Siegel and Castellan, 1988).

All analyses were done using Excel (Microsoft), ImageJ and STATISTICA 8.0 (StatSoft Inc., OK, USA).

3. Results

3.1 Behavioral Experiments

3.1.1. Memory persistence after strong and weak training protocols

The *Neohelice* memory model is based on the innate escape response of the crab to the threats of aerial predators (reviewed in detail in (Tomsic and Romano, 2013)). Repeated spaced presentation of a controlled visual danger stimulus (VDS) produces a reduction in the escape response to the VDS. Figure 1A summarizes the experimental procedure and replicates results previously described by Maldonado (2002). Figure 1A (Day 1) shows the escape response of crabs to the VDS throughout the 15 spaced trials of a strong-training protocol (TR) (Delorenzi, Pedreira, Romano, Pirola, Nahmod, and Maldonado, 1995; Tomsic and Romano, 2013). A control-untrained group (UN) received only one trial with the VDS. No difference was found for the first trial between TR and UN groups ($t(67)=1.23$, $p=0.22$) and a significant trial effect was disclosed for the trained group [F trial $(14, 462) = 8.067$; $p< 0.001$]. During the memory retention test one day and two days later (day 2 and day 3 respectively), both groups received one trial with the VDS. Strong trained crabs exhibit LTM revealed as a significant reduction of the escape response in comparison to the UN group [Day 2: $t\ (67) =3.35$, $p <0.01$; Day 3: $t\ (67) =2.03$, $p <0.05$] (Figure 1A, right panel).

A traditional strategy used to study the enhancing action of modulators on memory processes is to decrease the number of trials during training to induce a short-term memory that lasts only a few hours after training (McGaugh, 2000; Stough, Shobe, and Carew, 2006). Accordingly, after a weak training protocol consistent of 6 instead of 15 trials, crabs display memory up to 4 h after training, but not 24 h later (Delorenzi et al., 1995; Romano, Delorenzi, Pedreira, Tomsic, and Maldonado, 1996; Smal et al., 2011; Tomsic and Romano, 2013). However, we have recently shown that such weak training protocol does induce the formation of a LTM that depends on protein synthesis and mRNA transcription after acquisition but remains unexpressed (Delorenzi et al., 2014; Frenkel et al., 2010b). Reconsolidation experiments are crucial to disclose the existence of the unexpressed memory; in the figure 1Bi, we replicated the central experiment of our previous finding (Frenkel et al., 2005a), that as control was run here in parallel with the imaging experiments. Briefly, on day 1, untrained (UN) and trained (TR) groups of crabs were injected with vehicle (saline solution) and 15 minutes later underwent a weak training session (Fig 1B left panel and 1Bi). TR groups received 6 trials and UN groups received only 1 trial. A 2 x 6 ANOVA between the trained groups with repeated measures (trials) as second factor disclosed no significant differences between the two TR groups [Fgroup (1, 63) = 0.85; p = 0.36] and a significant trial effect [Ftrial (5, 315) = 11.94; p< 0.0001]. On day 2, one UN-TR pair was placed for 5-min in the training context (TC) without presentation of the VDS. The absence of the VDS during the reminder session triggers reconsolidation since there is a mismatch with the predictions generated by the re-activated memory (Frenkel et al., 2005a; Morris, Inglis, Ainge, Olverman, Tulloch, Dudai, and Kelly, 2006; Pedreira et al., 2004; Pedreira and Romano, 2013; Sevenster, Beckers, and Kindt, 2012). The second UN-TR pair was placed for 5-min in the training context and received 1 trial with the VDS (TC+VDS). The reinforced reminder avoids entering a reconsolidation process because there is no mismatch with the re-activated memory

(Pedreira and Romano, 2013). The TC+VDS pair confirmed that there was no memory expression 24 h after a weak training protocol (no significant differences between the UN and TR groups on Day 2, Fig. 1Bi; TR_{TC+VDS} vs. UN_{TC+VDS}, t(58) =-0.68; p = 0.59). Immediately after the reminder session, all animals were water deprived for 2 h. This treatment is a well-studied memory booster in crabs (Frenkel et al., 2002; Frenkel et al., 2005b). On Day 3, all animals were placed in the training context and tested with one VDS trial [ANOVA, a priori planned comparisons: F (3, 126)= 3.58, p=0.016]. The escape response showed no differences between TR_{TC+VDS} and UN_{TC+VDS} (p=0.26). Conversely, memory retention was disclosed for TR_{TC} vs. UN_{TC} groups (p< 0.01), in agreement with the interpretation that on day 2 a memory trace could be re-activated and memory could be enhanced by water deprivation during reconsolidation. No difference was found between untrained groups (UN_{TC} vs. UN_{TC+VDS}, p= 0.85). Thus, after weak training, even when memory expression is not observed (Day 2), reconsolidation is triggered on the condition that the presentation of the training context is not reinforced during the reminder session. These results replicate our previous findings (Frenkel et al., 2005a) showing that although the animals do not express the freezing behavior 24 h after weak training protocol, the memory persists and can be re-activated (Delorenzi et al., 2014; Frenkel et al., 2010b).

3.1.2. Pre-training administration of scopolamine impairs long-term memory expression

The muscarinic cholinergic receptor antagonist scopolamine has amnesic properties in both vertebrate and invertebrate, including crabs (Baratti, Boccia, and Blake, 2009; Beron de Astrada and Maldonado, 1999). However, we have recently shown that in *Neohelice* the amnesic effect of scopolamine is due to disruption of LTM expression rather than interference with mechanisms related with memory persistence (Caffaro et al., 2012). When scopolamine is injected immediately before or after training, there is a persistent memory trace that is not expressed in

the long term, but can be re-activated by appropriate reminders, similarly as it was shown above for the memory trace generated after a weak training protocol. Since one of the objectives in the present work was to search for neural correlates of memory, we invested effort in replicating the scopolamine's result obtained by Caffaro et al. 2012 but under training conditions that are used during neural recordings by calcium imaging. The training protocol used by Caffaro et al (2012), differs slightly from the standard one, in the fact that a change in the illumination of the training context anticipates in few seconds the stimulation with the VDS (Fustiñana, Carbo Tano, Romano, and Pedreira, 2013). In the present work, we adapted the experiment by Caffaro et al. 2012, replicating the same experimental design, but without the contextual cue that anticipates the VDS. This adaptation was performed to confirm that the interfering effect of scopolamine on LTM expression also takes place under the training conditions used in our current imaging experiments. The adaptation of the protocol and the validation of the previous result was a necessary condition to be able to compare behavioral and imaging results.

Scopolamine (SCP) 0.1μg/g was administered prior to training to two pairs of UN-TR groups. A 2 x 15 ANOVA (2 groups x 15 trials) with repeated measures on the second factor showed no significant differences between trained groups [Fgroup (1, 62) = 0.618; p = 0.43] and significant trial effect [Ftrial (13.07, 810.56) = 30.13; p< 0.0001, ε=0.93, Huynth-Feldt correction for sphericity violation] (Figure 1Bii, day 1, untrained groups are not shown). Memory was evaluated on Day 2 based on the escape response of animals that received the reinforced reminder (TC + VDS). As it was previously shown for animals injected with scopolamine prior to training (Beron de Astrada and Maldonado, 1999), no memory retention is observed despite the strong training [TR-SCP$_{TC+VDS}$ vs. UN-SCP$_{TC+VDS}$, t(62) = 0.34; p = 0.73] (Figure 1Bii, Day 2). The second pair of UN-TR groups received the unreinforced reminder that triggers reconsolidation (TC).

Immediately after the reminder session, all groups were water deprived for 2 hours. Scores at testing on Day 3, [ANOVA: F (3, 124) = 2.32, p= 0.07], showed no differences between the groups that had received the reinforced reminder on day 2 (TR-SCP_{TC+VDS} vs. UN-$SCP_{TC+VDS;}$ p=0.40) and no differences between the untrained groups (UN-SCP_{TC} vs. UN-SCP_{TC+VDS}, p= 0.77). Memory retention was disclosed on Day 3 among animals that had been exposed to conditions that trigger reconsolidation during the reminder session on day 2 (p< 0.05, for TR-SCP_{TC} vs. UN-SCP_{TC} group; Figure 1Bii, day 3). Thus, when scopolamine is administered before training, there is a persistent memory trace, which is not expressed, but is re-activated and labilized by the reminder that triggers reconsolidation. These results for scopolamine are in line with those of Caffaro et al. (2012) and validate the experimental setting that we use in the following calcium imaging experiments.

3.1.3. NMDAR antagonist MK801 impairs long-term memory formation

The activation of NMDAR is required for memory processes in several memory models (Mussig, Richlitzki, Rossler, Eisenhardt, Menzel, and Leboulle, 2010; Roberts and Glanzman, 2003; Tsien, Huerta, and Tonegawa, 1996; Wu, Xia, Fu, Wang, Chen, Leong, Chiang, and Tully, 2007). In *Neohelice*, pre-training administration of the NMDA receptor antagonist MK801 (*1 µg/g)* has amnesic effects without evident changes during training (Troncoso and Maldonado, 2002). We hypothesized that, in contrast to the effect of scopolamine 0.1 µg/g, but similar to the effects of cycloheximide, actinomycin and scopolamine 5 µg/g (Caffaro et al., 2012; Frenkel et al., 2010b), blockade of NMDA receptors interferes with mechanisms involved in the generation of a persistent memory . MK-801 (1µg/g) (MK801) was administered prior to training in two pairs of UN - TR groups. No differences were disclosed between TR groups during training (Figure 1Biii, Day 1, UN groups are not shown). A 2 x 15 ANOVA (2 groups x 15 trials) with

repeated measures on the second factor showed no significant differences between trained groups [F_{group} (1, 50) = 0.15; p = 0.69] and a significant trial effect [F_{trial}(9.26, 463) = 22.04; p< 0.0001, ε=0.66, Greenhouse-Geisser correction for sphericity violation]. As expected (Troncoso and Maldonado, 2002) the groups injected with MK-801 did not show memory retention at the reinforced reminder session (TR-MK_{TC+VDS} vs. UN-MK_{TC+VDS}, t(50) = 0.95; p = 0.34; Figure 1Biii, Day 2). Scores at testing on day 3 [ANOVA: F (3, 100) = 2.12, p= 0.10] showed no differences for this UN-TR pair, TR-MK_{TC+VDS} vs. UN-MK_{TC+VDS} (p=0.61) and no differences between untrained groups, UN-MK_{TC} vs. UN-MK_{TC+VDS} (p= 0.09), (Figure 1Biii, day 3). In this case, memory retention was neither disclosed for the TR-MK_{TC} group (p =0.68 for TR-MK_{TC} vs. UN-MK_{TC}). This result supports the hypothesis that the amnesic effect of blocking NMDA receptor is due to interference with mechanisms related with memory persistence, since we obtained no evidence of a memory trace that could be re-activated and labilized by the reminder on Day 2.

3.2. Learning-related neuronal plasticity in the medulla terminalis

In the decapod crustacean *Neohelice granulata,* the optic lobes (located within the eyestalks) comprise the *lamina*, *medulla*, *lobula* and *lateral protocerebrum* (Fig. 2A-D). The *lateral protocerebrum* comprises in turn several discrete neuropils including the highly interconnected *medulla terminalis* and the hemiellipsoid body (Derby and Blaustein, 1988; Sandeman, Sandeman, Derby, and Schmidt, 1992; Wolff, Harzsch, Hansson, Brown, and Strausfeld, 2012). The *medulla terminalis* - the most proximal structure to the central brain within the eyestalks of crayfish, lobsters, crabs and shrimps (Derby and Blaustein, 1988; McKinzie, Benton, Beltz, and Mellon, 2003; Sandeman, DeForest, and Wiese, 2002)- is a generic term used for several closely packed neuropils that receive inputs from the olfactory globular tract, the ipsi- and contralateral optic neuropils and the hemiellipsoid body (Blaustein et al., 1988; Sullivan and Beltz, 2001).

Based on the inputs from diverse sensory modalities, the *medulla terminalis* and the hemiellipsoid body -together with the accessory lobe in the central brain- have been largely considered higher-order integration centers potentially involved in high cognitive functions as learning and memory (Sandemam, Henning, and Harzsch, 2014). In order to measure neural activity in the medulla terminalis and evaluate learning-related neuronal plasticity during training and short-term memory test**,** we performed a mass staining of neurons in the medulla terminalis. We stabbed an electrode coated with the Ca2+ indicator dye Calcium Green™-1 dextran into a posterior–lateral position of the lateral protocerebrum (Figure 2D-E), on the opposite side of the hemiellipsoid body (Figure 2F), aiming at getting imaging recordings of Ca2+ signals from neurons in the dorsal area of the *medulla terminalis*. After calcium imaging, the optic lobes were dissected and visualized in a confocal microscope to verify that the stained and measured neuronal process were centered in the medulla terminalis neuropil (see 2.3.1., Materials and Methods)(Figure 2E,F).

3.2.1. Strong Training

We analyzed Ca2+ signals in response to the VDS throughout the CSM training protocol and in response to mechanical stimulation (MS) that was used as a control stimulus. Ca2+ imaging recordings were made throughout all trials of the training and the short-term memory test (see Figure 3 for details of the stimuli and sequence of the acquisition protocol). Responses to the VDS perceived through the contralateral eye and to the MS were recorded in the dorsal area of the *medulla terminalis.*

Figures 4A and B show measurements of calcium signals in a representative animal throughout training and short-term (30 min) testing trials. Figure 4A corresponds to false color pictures showing relative change in fluorescence (summation of % delta F/F), elicited by the first and

second cycle of the VDS and mechanical stimulus. As it is observed, the two cycles of each trial elicited distinguishable Ca2+ responses that are evident by the two peaks of activity (Figure 4B). The analysis of the Ca2+ signals was designed based on an autodefined AOI that covers all the region of the *medulla terminalis* in which a calcium response was above background activity in any of the recorded trials (2.3.3., Acquisition and data analysis of Calcium imaging, Material and Methods). Figure 4B shows the temporal detail of the calcium signal recorded in the AOI for the animal and trial respectively shown in Figure 4A. According to the postulate that the *medulla terminalis* receives inputs from diverse sensory modalities, , both, the VDS and the mechanical stimulus trigger Ca2+ responses. However, it is interesting to note at this point, that mechanical stimulus, unlike the VDS, does not elicit the escape response, thus supporting the interpretation that the signal measured in the medulla terminalis is not a mere predictor of animal's behavior (Supporting Information, Figure S2).

The summation of the average %deltaF/F of the AOI, throughout the frames of each VDS cycle and MS pulse, was used to analyze training related changes in Ca2+ signals (Figure 5). A two factors ANOVA was used to analyze differences in calcium signals between groups of crabs and between the first training trial and the test trial to evidence changes caused by training. No difference was found in the amplitude of the calcium signal elicited by the VDS among the 6 groups of crabs (first cycle $F(5,28)=0.45$, $p=0.81$; second cycle $F(5,28)=1.21$, $p=0.33$) showing that the different injections do not directly affect the amplitude of the calcium signals. In contrast, a statistically significant effect was found between response elicited in the first training trial and the test trial (first cycle $F(1,28)=4.88$, $p=0.035$; second cycle $F(1,28)=19.61$, $p<0.001$).

In the Strong-training group (15 trials) we found that Ca2+ signals in the *medulla terminalis* elicited by the VDS decreased during training and lasted until test trial. One-way repeated

measures ANOVA showed a trial effect for both VDS cycles: first cycle [Ftrial (3, 15) = 17.36, p<0.001], second cycle [Ftrial (3, 15) = 11.72, p<0.001]). Figure 4A shows a representative example of the reduction of the response along the training and during test trial (30 min after the end of training). The reduction in the calcium signal to the VDS were evident throughout the training session (Figure 5Ai, ii). For the test trial we found a significant difference compared to the first training trial for the second cycle (p<0.01), but not for the first cycle (p=0.27). This more pronounced decrease in calcium signals in the second cycle of the VDS trials is in accordance with changes previously described in lobula giant neurons responses (Tomsic et al., 2009). Surprisingly, we found an increase in Ca2+ signals elicited by the mechanical stimulus (control stimulus) in the short-term test (Figure 5Aiii, Figure 4A); first pulse MS [t(5) = -4.62, p<0.01], second pulse [t(5) = -3.76, p<0.05]. The opposite direction of the changes in the Ca2+ signals elicited by the VDS and the MS supports the interpretation that the changes were induced by training rather than by a mere time-dependent decrease of the signals such as changes in dye distribution, photobleaching or cytotoxicity. Moreover, the interpretation that the changes were induced by training is supported by the fact that no change wase found between the Ca2+ signals in the untrained group, neither for the VDS nor the MS (Figures 5Bi,ii,iii and examples in Supporting Information S3A,B); first VDS cycle [t(4)=-0.37, p=0.72], second cycle [t(4)=-0.49, p=0.65], first MS pulse [t(4) = -2.25, p=0.087], second pulse [t(4) =-1.14, p=0.32].

3.2.2. Weak Training

As it was shown in a previous section, a weak training protocol of 6 trials induces a long-term memory that is not expressed, but can be re-activated and labilized in the long term. It is possible that 6 trials are also not enough to induce the decrease in calcium signals showed after

15 trials. However, considering memory persistence and the possibility of a memory to be expressed as separate features of the memory trace, it is possible that the changes in the *medulla terminalis* are reflecting only one of these two features. To explore this possibility, we evaluate the learning-related changes induced by the weak training protocol. The Ca2+ signals from the *medulla terminalis* in response to the VDS showed an evident trial effect (Figures 5Ci, ii and S3C,D)(one-way repeated measures ANOVA trials 1,6 and testing: first VDS cycle [F_{trial} (2, 10) = 10.29, $p<0.01$] and second cycle [F_{trial} (2, 10) = 20.20, $p<0.001$]). We found differences between trial 1 and trial 6, and in case of the testing trial a significant difference for the second cycle ($p<0.01$), but not the first one ($p=0.07$). Thus, similar to the strong training protocol, the weak training induced both a reduction in the neuronal activity elicited by VDS 30 minutes after training and an increase in the activity triggered by the mechanical stimulus (Figure 5Biii); first pulse MS [$t(5) = 5.52$, $p<0.01$] and second pulse [$t(5) = -3.61$, $p<0.05$].

3.2.3. Effect of scopolamine on training induced changes in the medulla terminalis

Scopolamine 0.1 µg/g allows the formation of a persistent memory trace that is not expressed but can be re-activated and labilized (Figure 1Bii and (Caffaro et al., 2012)). In a group of animals injected with scopolamine 0.1 µg/g before a strong training protocol, Ca2+ signals from the *medulla terminalis* in response to VDS showed a significant trial effect (Figures 5Di,ii and Supplemental Material S4A,B) (one-way repeated measures ANOVA trails 1, 6, 15 and test trial : first cycle [F_{trial} (3, 12) = 5.75, $p<0.05$], second cycle [F_{trial} (3, 12) = 7.49, $p<0.01$]). Figure 5D shows that the Ca2+ response in trials 6, 15 and test trial was lower than in trial 1 for the first and second cycles of the VDS ($p<0.01$ in all cases). Similar to strong and weak training groups injected with vehicle, we found a reduction in the neuronal activity elicited by the VDS after

training and an increase for the MS that persist at least 30 minutes after training (Figure 5Diii); first pulse MS [$t(3) = -2.54$, $p=0.08$], second pulse [$t(3) = -7.09$, $p<0.01$].

In contrast to the previous experiment, scopolamine at a high dose of 5 μg/g is known to prevent the formation of a persistent memory trace. Crabs neither show long term memory expression nor evidence of memory reactivation when this dose of scopolamine is administered before training (Caffaro et al., 2012). In the next experiment we treated a group of animals with scopolamine 5 μg/g before the strong training protocol, and measured Ca2+ signals from the *medulla terminalis*. At difference with all cases reported above, the Ca2+ responses to the VDS along the training and testing did not show a trial effect in animals trained under effect of scopolamine 5μg/g (Figure 5Ei,ii and Supporting Information S4 C,D)(one-way repeated measures ANOVA trails 1,6, 15 and test: first VDS cycle [$F_{trial}(1.14, 5.71) = 2.87$, $p=0.14$, $\varepsilon=0.38$, Greenhouse-Geisser correction for sphericity violation], second cycle [$F_{trial}(3, 15) = 2.92$, $p=0.07$]). A significant decrease was found only in trial 15 (first cycle, $p<0.05$; second cycle, $p<0.05$). The reduction of the Ca2+ response in the fifteenth trial did not persist until the test trial 30 minutes after training; first cycle, $p=0.79$; second VDS cycle, $p=0.06$. Thus, the reduction in the neuronal activity elicited by VDS observed 30 minutes after the end of training was blocked by scopolamine 5 μg/g, correlating with the behavioral evidence of an interference effect in the persistence of CSM after this treatment (Caffaro et al., 2012). In regard to MS, we found the same increase that we described in the three previous groups 30 min after the end of training (Figure 5Eiii); first pulse MS [$t(5) = -7.68$, $p<0.001$], second pulse [$t(5) = -3.91$, $p<0.05$].

3.2.4. NMDAR antagonist MK-801 blocks training induced changes in the medulla terminalis.

Comparable to scopolamine 5 μg/g, actinomycin and cycloheximide (Caffaro et al., 2012; Frenkel et al., 2010b), here we show that MK-801 prevents the formation of a persistent memory

trace, crabs do neither show long-term memory expression nor evidence of memory re-activation after this treatment (Figure 1Biii). In the next experiment, we studied the effect of MK-801 in regard to the changes in calcium signals described above. In a group of animals injected with MK-801 (*1 µg/g)* before a strong training protocol, we found a trial effect for both VDS cycles (one-way repeated measures ANOVA: first cycle [Ftrial (3, 15) = 6.62, $p<0.01$], second VDS cycle [Ftrial (3, 15) = 4.72, $p<0.05$]); Ca2+ signals from the *medulla terminalis* in response to VDS showed a reduction throughout the training session (Figures 4C,D and 5Fi,ii). However, the reduction of the Ca2+ responses did not persist until the short-term test. No difference was found between trial 1 and test trial neither for the first ($p=0.93$) nor for the second VDS cycle ($p=0.42$). Thus, the reduction in the neuronal activity elicited by VDS observed 30 minutes after the end of a strong training was blocked by MK-801. Moreover, in regard to calcium responses elicited by the MS, MK-801 was the only treatment which blocked the increase in the Ca2+ signals (Figure 5Fiii); first pulse MS [$t(4) = -0.99$, $p=0.38$], second pulse [$t(4) = 2.28$, $p=0.08$].

3.3 Short-term memory expression

In parallel to the imaging experiments described in the previous section, we ran behavioral experiments to measure memory expression 30 minutes after training in order to establish in what extent short term memory is correlated with the changes recorded in the *medulla terminalis*. For all experimental groups, including weak-trained animals and strong-trained animals with different pharmacological treatments, we found a significant reduction in escape response across training trials (Supporting Information, Figure S5). In particular, we noted that in the group that underwent strong training with scopolamine 5µg/g, the decrease in escape response during training is notably lower than for others treatments (Figure S5iv). This effect has not

been found in Caffaro et al. (2012), most likely because in that work, animals were trained with a slightly different protocol in which a change in context illumination anticipated the training trial producing a stronger associative strength between context and VDS (Fustiñana et al., 2013). Remarkably, short term memory in the test trial 30 minutes after training was disclosed in all groups. These results suggest that short-term memory expression is not reflected by the changes in the Ca2+ responses analyzed in the *medulla terminalis*.

4. Discussion

Here, we confirmed previous results and obtained new ones supporting the hypothesis that memory persistence and memory expression constitute different mechanistic features of the memory trace. Furthermore, we found that training induces changes in the neuronal responses to the learned stimuli in the *medulla terminalis,* which correlate with the persistence of the memory but not with its probability to be expressed in the long term or even in the short term (Table 1). Thus, the *medulla terminalis* would house, or at least reflects, part of the forming memory trace that is related to memory persistence.

4.1. Memory persistence, memory re-activation and memory expression

Memory processes and their neuronal correlates have generally been examined on the premise that retrieval and memory expression are interchangeable concepts. However, in previous (Delorenzi et al., 2014) and in the present study, we provide evidence of the existence of memories that can be re-activated even though they are not behaviorally expressed. The design of reconsolidation experiments was critical to bring to light the re-activation of unexpressed memories, as it was done in the behavioral experiments from Figure 1 and were original

performed by Frenkel et al. (2015). What we evaluated at the test session on the third day of these experiments was whether a memory that was not expressed on the second day, could be re-activated and became labile by the presentation of a reminder (Delorenzi et al., 2014). This strategy allowed us to separate unexpressed consolidated memories from memories that apparently do not exist (Figure 1Biii). In the present study, we got more evidence of memories that are retrieved without being expressed. The proof that they were retrieved is that memory has been re-activated and labilized to enter the reconsolidation phase (Lewis, 1979; Misanin, Miller, and Lewis, 1968; Pedreira and Romano, 2013; Przybyslawski and Sara, 1997; Rodriguez-Ortiz, Balderas, Garcia-Delatorre, and Bermudez-Rattoni, 2012; Sara, 2000). Remarkably, reconsolidation depends on a mismatch between actual and expected experiences during the reminder session. In *Neohelice* the effects of amnesic or memory enhancing agents administered after LTM re-activation are not observed if the reminder is reinforced by the presentation of the VDS (i.e. no mismatch condition at reminder session)(Pedreira and Romano, 2013). This mismatch condition (termination of the reminder session without the predicted reinforcement) is necessary to observe the enhancing effect of water deprivation after memory re-activation. Concordantly, when the reminder is reinforced, thus fulfilling memory expectations, the memory trace is not labilized and no improvement in LTM is observed (Figures 1Bi, ii)(Delorenzi et al., 2014; Frenkel et al., 2005a). These results provides the essential evidence that the memory enhancement cannot be explained as the summation of sub-threshold memory traces or the recovery of failures in the re-activation of the memory trace (Caffaro et al., 2012; Gold, 2006; Hardt, Wang, and Nader, 2009; Squire, 2006). By using this strategy, here we corroborate our previous results and extend them to the more standard training conditions of CSM, showing that both weak training and the amnesic agent scopolamine (0.1 µg/g) administered before a strong training allow the formation of LTM (Figure 1Bi. ii) which is not

expressed, but can be retrieved and reconsolidated (Caffaro et al., 2012). In contrast, and as it was previously shown with cycloheximide, actinomycine and scopolamine 5 μg/g, here we found that the administration of the NMDA receptor blocker MK801 obstructs the formation of an reactivatable memory trace (Figure 1Biii). In conclusion, the results show that the mechanisms involved in memory persistence and the mechanisms involved in the long-term memory expression can be pharmacologically separated. This view can explain, for instance, recent results showing that LTM may persist covertly after its apparent elimination by some antimnemonic treatments (Chen, Cai, Pearce, Sun, Roberts, and Glanzman, 2014; Ryan, Roy, Pignatelli, Arons, and Tonegawa, 2015).

4.2. The *medulla terminalis*

The *medulla terminalis* neuropil is highly interconnected with the hemiellipsoid body and with several neuropils from the central brain and the ipsi and contralateral optic lobes. *Ex-vivo* intracellular recordings and several neuroanatomical studies in other decapods substantiate that the *medulla terminalis*/hemiellipsoid body complex integrates multimodal sensory inputs (Derby and Blaustein, 1988; McKinzie et al., 2003; Sandeman et al., 2002; Sandeman et al., 1992; Sullivan and Beltz, 2001; 2005; Wolff et al., 2012; Yagodin, Collin, Alkon, Sheppard, and Sattelle, 1999). In accordance, we found in-vivo calcium signals elicited by both mechanical and visual stimulation in the *medulla terminalis*. The specific neuronal inputs that are responsible for the responses to the contralateral visual stimulation and mechanical stimulation still need to be explored. One candidate are the well-documented lobula giant neurons which also respond to mechanical and visual inputs, integrate binocular visual information and reduce their response to the VDS during training. The duration of the decrement induced by a strong training in the response of these neurons correlates strikingly with the changes in the escape response to the VDS (Beron de

Astrada, Tuthill, and Tomsic, 2009; Sztarker and Tomsic, 2004; Tomsic et al., 2009; Tomsic and Romano, 2013). The other principal neural structure of the lateral protocerebrum that feeds the *medulla terminalis* is the hemiellipsoid body. Although the hemiellipsoid body is believed to be a center of high order integration that plays a role in memory processes, no direct experimental evidence has been yet obtained in support of this view (McKinzie et al., 2003; Schachtner, Schmidt, and Homberg, 2005; Wolff and Strausfeld, 2016). In Malacostraca -crayfish and lobsters for instance- a type of neuron called parasol cells were described as providing the principal output channels from the hemiellipsoid body to the *medulla terminalis*. Parasol cells have multisensory tuning, and response properties that closely match those of mushroom body output neurons in insects (Loesel, Wolf, Kenning, Harzsch, and Sombke, 2013; McKinzie et al., 2003; Sandeman et al., 2002; Schachtner et al., 2005; Strausfeld, Hansen, Li, Gomez, and Ito, 1998; Sullivan and Beltz, 2005; Wolff et al., 2012). These cells are interesting candidates to look for neural correlates of memory since they receive indirect olfactory, tactile and contralateral visual sensory input via brain projection neurons and also input from visual ganglia in the ipsilateral optic lobe. The changes induced by training in the Ca2+ signals in the *medulla terminalis* that we showed here (Figures 4, 5 and Supporting Information) represent the first *in vivo* experimental evidence that supports the hypothesis that the neuropils in the lateral protocerebrum play a role in memory (Sandemam et al., 2014; Sandeman et al., 2002; Yagodin et al., 1999). In addition, we did not find significant differences in the spatial patterns of Ca2+ signal triggered by different sensory inputs in the *medulla terminalis* (Supporting Information, Figure S1). This suggests that the recorded activity do not encode the stimulus itself. Consequently, and although speculative, the activity in *medulla terminalis* might reflect the activity of downstream output neurons after the interaction of the signaled stimulus with the hemiellipsoid bodies that, like insect mushroom bodies, perhaps have a broader role in regulating the animal internal state (Aso, Sitaraman,

Ichinose, Kaun, Vogt, Belliart-Guerin, Placais, Robie, Yamagata, Schnaitmann, Rowell, Johnston, Ngo, Chen, Korff, Nitabach, Heberlein, Preat, Branson, Tanimoto, and Rubin, 2014; Strausfeld et al., 1998). The fact that both VDS and mechanical stimulus types elicit calcium signals but, in the present experimental conditions, only the VDS elicits an escape response denote that multimodal neurons would be present in the *medulla terminalis*. However, the response to different sets of inputs may occur only in coordination with other higher integrative centers, mainly accessory lobes and hemiellipsoid body, that altogether may be responsible for a highly selective filtering of sets of environmental inputs that trigger appropriate behaviors (Sandemam et al., 2014). Our current imaging experiments are now focused on studying changes related to learning and memory in the hemiellipsoid body (Figure 2F) upon the vision that it is a place where a relevant part of engram might be formed.

4.3. Neural correlates of memory persistence in the medulla terminalis

We used *in vivo* calcium imaging to explore putative correlates of CSM traces in the *medulla terminalis* during and after different training protocols. We found that Ca2+ signals triggered by the VDS decreased during strong training protocols and lasted at least 30 min after training. Such change in the *medulla terminalis* after the end of training correlates with memory persistence independently of the probability of this memory to be expressed. This conclusion is based on the observation that in all cases in which the animals received a treatment that generates a long-term memory that remains unexpressed, the changes in the *medulla terminalis* turned out to be similar to those induced by a strong training protocol that induces a memory that is expressed in the long term. The change was specifically observed in other two situations: a weak training protocol or when animals received scopolamine 0.1ug/g, together with a strong training (Figure 5A, C, D). The muscarinic cholinergic antagonist scopolamine impairs memory

in vertebrates and invertebrates (Baratti et al., 2009; Beron de Astrada and Maldonado, 1999; Buccafusco, 2009; da Silva, Silva Martins, Linck Vde, Herrmann, Mai, Nunes, and Elisabetsky, 2009; Quirarte, Cruz-Morales, Cepeda, Garcia-Montanez, Roldan-Roldan, and Prado-Alcala, 1994; Weinberger, 2006). As aforementioned, weak training protocols or scopolamine (0.1 μg/g) administration combined with strong training, induce the formation of a persistent CSM trace that is not expressed in the long term but can be re-activated and labilized (Figures 1Bii). The analysis of the Ca2+ signals in the *medulla terminalis* showed a striking parallel with the persistence of memory in the two mentioned cases. Similarly to strongly trained crabs, we found a decrease in Ca2+ signals 30 minutes after a weak training protocol or after a strong training with scopolamine 0.1ug/g (Figures 5C, D, and Supporting Information S3 C,D, S4A,B). Furthermore, upon these treatments we also found the increase in the response to mechanical stimulus at testing (Figure 5C, D iii). Unlike the long-term memory test, we found a significant reduction in the behavioral escape response during short-term testing (Supporting Information Figure S5).

In support to the view that the change in the *medulla terminallis* activity is related with memory persistence, we found no reduction of the Ca2+ signals at testing trial in the groups of animals in which the pharmacological treatments blocked the formation of long-term memory (Figure 5F,E). This was the case for a high dose of scopolamine (5 μg/g) (Caffaro et al., 2012) and the NMDAR blocker MK-801 (Figure 1Biii) which appears to affect the consolidation process (see (Delorenzi et al., 2014) for a critical view). Moreover, the NMDAR antagonist also blocked the increase in the calcium response to mechanical stimulus induced by training (Figure 5Fiii). This result, together with the described localization of NMDAR in the lateral protocerebrum of *Neohelice(Hepp, Tano, Pedreira, and Freudenthal, 2013)*, add evidence to the proposed role in

memory processes of these neuropils (Blaustein et al., 1988). Regarding muscarinic receptors, they have been described in crustaceans' central nervous system, eyestalk ganglia included (Barker, Murray, Siebenaller, and Mpitsos, 1986) but to the best of our knowledge there is no precise descrption in the lateral protocerebrum. However, these receptors have recognized functions in the visual system of crustaceans (Pfeiffer and Glantz, 1989) and in insects play a role in several neuropils, mushrooms bodies included (Silva, Molina-Fernandez, Ugalde, Tognarelli, Angel, and Campusano, 2015).

4.4. The changes in medulla terminalis reflect long- but not short-term memory

All trained groups showed a reduction in the escape response tested 30 min after training (FigureS5) irrespective of the training strength or the amnesic treatment they had received. It has been shown that this short-term memory involves the modulatory action of endogenous opioids (Romano, Lozada, and Maldonado, 1991; Tomsic et al., 1991). Indeed, during this short period after learning, in crabs and several models behavioral memory can be updated with new contextual information (Smal et al., 2011; Suarez, Smal, and Delorenzi, 2010). The transfer from short, intermediate and long-term memory is not a simply sequential events and includes simultaneous/parallel processes (Izquierdo, Cammarota, Medina, and Bevilaqua, 2004; Menzel, 1999; Stough et al., 2006). Short-term behavioral response can be highly dominated by arousal, sensitization or habituation processes (Gerber and Menzel, 2000; Menzel, 1999; Tomsic et al., 2009). Thus, short-term memory expression can be unspecific and imprecise to a certain extent. The association components of CSM develop and express themselves later at behavioral level, after the consolidation period has finalized (Suarez et al., 2010). By contrasting the calcium signals and behavioral results at the short term (Table 1), the present data strongly suggests that

the neural mechanisms involved in the reduction of the behavioral response in the short term are different from and independent of the changes in the *medulla terminalis* described here.

5. Conclusions

In summary, after training, we found a specific reduction of the Ca2+ signals elicited by the VDS in the *medulla terminalis*. Unlike treatments that lead to persistent, re-activatable, but not expressed long-term memory, the amnesic agents MK-801 and scopolamine at dose 5µg/g blocked the Ca2+ signals decrement in the short-term test. These changes in the *medulla terminalis* Ca2+ signals may represent the development of a component of the memory trace related to the potential to be re-activated, but not to its long-term expression. Thus, the results constitute physical evidence in favor of the hypothesis that memory persistence and the probability to express this memory might involve different and separate components of the memory trace.

Acknowledgements

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina (CONICET) and Universidad de Buenos Aires. Grants: UBA: 20020110100071; CONICET: PIP 11220120100170CO; ANPCyT PICT-2013-1020. We thank G. Galizia for helpful comments about the manuscript. We thank professor C.M. Baratti for the scopolamine, and Angel Vidal and José Clemente for technical assistance.

Abbreviations

CSM context-signal memory

LTM long-term memory

MS mechanical stimulus

VDS visual danger stimulus

NMDA N-methyl-D-aspartate; (NMDAR) receptors.

References

Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K. R., Vogt, K., Belliart-Guerin, G., Placais, P. Y., Robie, A. A., Yamagata, N., Schnaitmann, C., Rowell, W. J., Johnston, R. M., Ngo, T. T., Chen, N., Korff, W., Nitabach, M. N., Heberlein, U., Preat, T., Branson, K. M., Tanimoto, H., & Rubin, G. M. (2014). Mushroom body output neurons encode valence and guide memory-based action selection in Drosophila. *Elife, 3*.

Baratti, C. M., Boccia, M. M., & Blake, M. G. (2009). Pharmacological effects and behavioral interventions on memory consolidation and reconsolidation. *Braz.J.Med.Biol.Res., 42*, 148-154.

Barker, D. L., Murray, T. F., Siebenaller, J. F., & Mpitsos, G. J. (1986). Characterization of muscarinic cholinergic receptors in the crab nervous system. *J Neurochem, 46*, 583-588.

Barreiro, K. A., Suarez, L. D., Lynch, V. M., Molina, V. A., & Delorenzi, A. (2013). Memory expression is independent of memory labilization/reconsolidation. *Neurobiol Learn Mem, 106C*, 283-291.

Beron de Astrada, M., & Maldonado, H. (1999). Two related forms of long-term habituation in the crab Chasmagnathus are differentially affected by scopolamine. *Pharmacol.Biochem.Behav., 63*, 109-118.

Beron de Astrada, M., Tuthill, J. C., & Tomsic, D. (2009). Physiology and morphology of sustaining and dimming neurons of the crab Chasmagnathus granulatus (Brachyura: Grapsidae). *J Comp Physiol A Neuroethol Sens Neural Behav Physiol, 195*, 791-798.

Blaustein, D. N., Derby, C. D., Simmons, R. B., & Beall, A. C. (1988). Structure of the Brain and Medulla Terminalis of the Spiny Lobster Panulirus argus and the Crayfish Procambarus clarkii, with an Emphasis on Olfactory Centers. *Journal of Crustacean Biology, 8*, 493-519.

Buccafusco, J. J. (2009). The Revival of Scopolamine Reversal for the Assessment of Cognition-Enhancing Drugs.

Caffaro, P. A., Suarez, L. D., Blake, M. G., & Delorenzi, A. (2012). Dissociation between memory reactivation and its behavioral expression: scopolamine interferes with memory expression without disrupting long-term storage. *Neurobiol Learn Mem, 98*, 235-245.

Chen, S., Cai, D., Pearce, K., Sun, P. Y., Roberts, A. C., & Glanzman, D. L. (2014). Reinstatement of long-term memory following erasure of its behavioral and synaptic expression in Aplysia. *Elife, 3*.

Chinga, G., & Syverud, K. (2007). Quantification of paper mass distributions within local picking areas. *Nordic Pulp and Paper Res. J., 22*, 441-446.

da Silva, A. L., Silva Martins, B., Linck Vde, M., Herrmann, A. P., Mai, N., Nunes, D. S., & Elisabetsky, E. (2009). MK801- and scopolamine-induced amnesias are reversed by an Amazonian herbal locally used as a "brain tonic". *Psychopharmacology (Berl), 202*, 165-172.

Davis, R. L. (2011). Traces of Drosophila memory. *Neuron, 70*, 8-19.

Delorenzi, A., Maza, F. J., Suarez, L. D., Barreiro, K., Molina, V. A., & Stehberg, J. (2014). Memory beyond expression. *J Physiol Paris, 108*, 307-322.

Delorenzi, A., Pedreira, M. E., Romano, A., Pirola, C. J., Nahmod, V. E., & Maldonado, H. (1995). Acute administration of angiotensin II improves long-term habituation in the crab Chasmagnathus. *Neurosci Lett, 196*, 193-196.

Derby, C. D., & Blaustein, D. N. (1988). Morphological and physiological characterization of individual olfactory interneurons connecting the brain and eyestalk ganglia of the crayfish. *J.Comp Physiol [A], 163*, 777-794.

Dudai, Y. (2002a). *Memory from A to Z*: Oxford University Press.

Dudai, Y. (2002b). Molecular bases of long-term memories: a question of persistence. *Curr.Opin.Neurobiol., 12*, 211-216.

Dudai, Y., & Morris, R. G. (2013). Memorable trends. *Neuron, 80*, 742-750.

Frenkel, L., Dimant, B., Portiansky, E. L., Imboden, H., Maldonado, H., & Delorenzi, A. (2010a). Neuroanatomical distribution of angiotensin-II-like neuropeptide within the central nervous system of the crab Chasmagnathus; physiological changes triggered by water deprivation. *Cell Tissue Res, 341*, 181-195.

Frenkel, L., Freudenthal, R., Romano, A., Nahmod, V. E., Maldonado, H., & Delorenzi, A. (2002). Angiotensin II and the transcription factor Rel/NF-kappaB link environmental water shortage with memory improvement. *Neuroscience, 115*, 1079-1087.

Frenkel, L., Maldonado, H., & Delorenzi, A. (2005a). Memory strengthening by a real-life episode during reconsolidation: an outcome of water deprivation via brain angiotensin II. *Eur J Neurosci, 22*, 1757-1766.

Frenkel, L., Maldonado, H., & Delorenzi, A. (2005b). Retrieval improvement is induced by water shortage through angiotensin II. *Neurobiol Learn Mem, 83*, 173-177.

Frenkel, L., Suarez, L. D., Maldonado, H., & Delorenzi, A. (2010b). Angiotensin modulates long-term memory expression but not long-term memory storage in the crab Chasmagnathus. *Neurobiol Learn Mem, 94*, 509-520.

Fustiñana, M. S., Carbo Tano, M., Romano, A., & Pedreira, M. E. (2013). Contextual Pavlovian conditioning in the crab Chasmagnathus. *Anim Cogn, 16*, 255-272.

Galizia, C. G., & Vetter, R. S. (2004). Optical Methods for Analyzing Odor-Evoked Activity in the Insect Brain, *Methods in Insect Sensory Neuroscience*: CRC Press.

Genovese, G., Senek, M., Ortiz, N., Regueira, M., Towle, D. W., Tresguerres, M., & Luquet, C. M. (2006). Dopaminergic regulation of ion transport in gills of the euryhaline semiterrestrial crab Chasmagnathus granulatus: interaction between D1- and D2-like receptors. *J Exp Biol, 209*, 2785-2793.

Gerber, B., & Menzel, R. (2000). Contextual modulation of memory consolidation. *Learn Mem., 7*, 151-158.

Gold, P. E. (2006). The many faces of amnesia. *Learn Mem.2006 Sep-Oct;13(5):506-14, 13*, 506-514.

Hardt, O., Wang, S. H., & Nader, K. (2009). Storage or retrieval deficit: the yin and yang of amnesia. *Learn Mem., 16*, 224-230.

Hepp, Y., Tano, M. C., Pedreira, M. E., & Freudenthal, R. A. (2013). NMDA-like receptors in the nervous system of the crab Neohelice granulata: a neuroanatomical description. *J Comp Neurol, 521*, 2279-2297.

Hoeger, U., & Florey, E. (1989). Catecholamine degradation in the hemolymph of the chinese crab, Eriocheir sinensis *Comp Biochem.Physiol.C, 92*, 323-327.

Howell, D. C. (1987). *Statistical methods for psychology*. Boston: Duxbury.

Izquierdo, I., Cammarota, M., Medina, J. H., & Bevilaqua, L. R. (2004). Pharmacological findings on the biochemical bases of memory processes: a general view. *Neural Plast., 11*, 159-189.

Lewis, D. J. (1979). Psychobiology of active and inactive memory. *Psychol Bull, 86*, 1054-1083.

Loesel, R., Wolf, O. T., Kenning, M., Harzsch, S., & Sombke, A. (2013). Architectural principles and evolution of the arthropod central nervous system, in Arthropod Biology and Evolution. *In* A. Minelli, G. Boxshall, & G. Fusco (Eds.), *Arthropod Biology and Evolution* (pp. 299-342). Berlin, Heidelberg: Springer.

Loftus, G. R., & Masson, M. E. (1994). Using confidence intervals in within-subject designs. *Psychon Bull Rev, 1*, 476-490.

Lukowiak, K., Sangha, S., Scheibenstock, A., Parvez, K., McComb, C., Rosenegger, D., Varshney, N., & Sadamoto, H. (2003). A molluscan model system in the search for the engram. *J.Physiol Paris, 97*, 69-76.

Maldonado, H. (2002). Crustaceans as models to investigate memory illustrated by extensive behavioral and physiological studies in Chasmagnathus. *In* K.Wiese (Ed.), *The crustacean nervous system* (pp. 314-327). Berlin: Springer.

Maldonado, H., Romano, A., & Tomsic, D. (1997). Long-term habituation (LTH) in the crab Chasmagnathus: a model for behavioral and mechanistic studies of memory. *Braz.J.Med.Biol.Res., 30*, 813-826.

McGaugh, J. L. (2000). Memory--a century of consolidation. *Science, 287*, 248-251.

McKinzie, M. E., Benton, J. L., Beltz, B. S., & Mellon, D. (2003). Parasol cells of the hemiellipsoid body in the crayfish Procambarus clarkii: dendritic branching patterns and functional implications. *J.Comp Neurol., 462*, 168-179.

Menzel, R. (1999). Memory dynamics in the honeybee. *J.Comp Physiol A, 185*, 323-340.

Menzel, R. (2014). The insect mushroom body, an experience-dependent recoding device. *J Physiol Paris, 108*, 84-95.

Misanin, J. R., Miller, R. R., & Lewis, D. J. (1968). Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace. *Science, 160*, 554-555.

Morris, R. G., Inglis, J., Ainge, J. A., Olverman, H. J., Tulloch, J., Dudai, Y., & Kelly, P. A. (2006). Memory reconsolidation: sensitivity of spatial memory to inhibition of protein synthesis in dorsal hippocampus during encoding and retrieval. *Neuron, 50*, 479-489.

Morris, R. G. M. (2007). Memory: Distinctions and dilemmas. *In* H. L. Roedriger, Y. Dudai, & S. M. Fitzpatrick (Eds.), *Science of Memory: Concepts* (pp. 29-34). Great Britain: Oxford University Press, Inc.

Mussig, L., Richlitzki, A., Rossler, R., Eisenhardt, D., Menzel, R., & Leboulle, G. (2010). Acute disruption of the NMDA receptor subunit NR1 in the honeybee brain selectively impairs memory formation. *J Neurosci, 30*, 7817-7825.

Pedreira, M. E., Dimant, B., & Maldonado, H. (1996). Inhibitors of protein and RNA synthesis block context memory and long-term habituation in the crab Chasmagnathus. *Pharmacol.Biochem.Behav., 54*, 611-617.

Pedreira, M. E., Perez-Cuesta, L. M., & Maldonado, H. (2004). Mismatch between what is expected and what actually occurs triggers memory reconsolidation or extinction. *Learn.Mem., 11*, 579-585.

Pedreira, M. E., & Romano, A. (2013). Memory Reconsolidation and Extinction in Invertebrates: Evolutionarily Conserved Characteristics of Memory Reprocessing and Restabilization. *In* C. M. Alberini (Ed.), *Memory Reconsolidation* (pp. 139-164). San Diego, CA, USA: Academic Press.

Pereyra, P., Gonzalez, P. E., & Maldonado, H. (2000). Long-lasting and context-specific freezing preference is acquired after spaced repeated presentations of a danger stimulus in the crab Chasmagnathus. *Neurobiol.Learn.Mem., 74*, 119-134.

Perez-Cuesta, L. M., & Maldonado, H. (2009). Memory reconsolidation and extinction in the crab: mutual exclusion or coexistence? *Learn Mem, 16*, 714-721.

Pfeiffer, C., & Glantz, R. M. (1989). Cholinergic synapses and the organization of contrast detection in the crayfish optic lobe. *J Neurosci, 9*, 1872-1882.

Przybyslawski, J., & Sara, S. J. (1997). Reconsolidation of memory after its reactivation. *Behav.Brain Res., 84*, 241-246.

Quirarte, G. L., Cruz-Morales, S. E., Cepeda, A., Garcia-Montanez, M., Roldan-Roldan, G., & Prado-Alcala, R. A. (1994). Effects of central muscarinic blockade on passive avoidance: anterograde amnesia, state dependency, or both? *Behav Neural Biol, 62*, 15-20.

Rescorla, R. A. (1988). Behavioral studies of Pavlovian conditioning. *Annu Rev Neurosci, 11*, 329-352.

Roberts, A. C., & Glanzman, D. L. (2003). Learning in Aplysia: looking at synaptic plasticity from both sides. *Trends in Neurosciences, 26*, 662-670.

Rodriguez-Ortiz, C. J., Balderas, I., Garcia-Delatorre, P., & Bermudez-Rattoni, F. (2012). Taste aversion memory reconsolidation is independent of its retrieval. *Neurobiol Learn Mem*.

Romano, A., Delorenzi, A., Pedreira, M. E., Tomsic, D., & Maldonado, H. (1996). Acute administration of a permeant analog of cAMP and a phosphodiesterase inhibitor improve long-term habituation in the crab Chasmagnathus. *Behav Brain Res, 75*, 119-125.

Romano, A., Locatelli, F., Freudenthal, R., Merlo, E., Feld, M., Ariel, P., Lemos, D., Federman, N., & Fustinana, M. S. (2006). Lessons from a crab: molecular mechanisms in different memory phases of Chasmagnathus. *Biol.Bull., 210*, 280-288.

Romano, A., Lozada, M., & Maldonado, H. (1991). Nonhabituation processes affect stimulus specificity of response habituation in the crab Chasmagnathus granulatus. *Behav Neurosci, 105*, 542-552.

Rosenthal, R., & Rosnow, R. L. (1985). *Contrasts analysis focused comparisons in the analysis of variance*. Cambridge, UK: Cambridge UP.

Ryan, T. J., Roy, D. S., Pignatelli, M., Arons, A., & Tonegawa, S. (2015). Memory. Engram cells retain memory under retrograde amnesia. *Science, 348*, 1007-1013.

Sandemam, D. C., Henning, M., & Harzsch, S. (2014). Adaptive Trends in Malacostracan Brain Form and Function Related to Behavior. *In* C. D. Derby, & M. Thiel (Eds.), *Nervous Systems and Control of Behavior* (pp. 11-45). Ney York: Oxford Univesrity Press.

Sandeman, D., DeForest, M., Jr., & Wiese, K. (2002). Olfactory centers in the brain of freshwater crayfish, *The Crustacean Nervous System*. Berlin Heidelberg: Springer Verlag.

Sandeman, D., Sandeman, R., Derby, C., & Schmidt, M. (1992). Morphology of the brain of crayfish, crabs, and spiny lobsters: a common nomenclature for homologous structures. *Biol.Bull.*, 304-326.

Sara, S. J. (2000). Retrieval and reconsolidation: toward a neurobiology of remembering. *Learn.Mem., 7*, 73-84.

Schachtner, J., Schmidt, M., & Homberg, U. (2005). Organization and evolutionary trends of primary olfactory brain centers in Tetraconata (Crustacea+Hexapoda). *Arthropod Struct Dev, 34*, 257-299.

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat Meth, 9*, 671-675.

Sevenster, D., Beckers, T., & Kindt, M. (2012). Retrieval per se is not sufficient to trigger reconsolidation of human fear memory. *Neurobiol Learn Mem*.

Siegel, S., & Castellan, N. J. (1988). *Nonparametric Statistics for the Behavioral Sciences*: McGraw-Hill.

Silva, B., Molina-Fernandez, C., Ugalde, M. B., Tognarelli, E. I., Angel, C., & Campusano, J. M. (2015). Muscarinic ACh Receptors Contribute to Aversive Olfactory Learning in Drosophila. *Neural Plast, 2015*, 658918.

Smal, L., Suarez, L. D., & Delorenzi, A. (2011). Enhancement of long-term memory expression by a single trial during consolidation. *Neurosci Lett, 487*, 36-40.

Squire, L. R. (2006). Lost forever or temporarily misplaced? The long debate about the nature of memory impairment. *Learn Mem, 13*, 522-529.

Stough, S., Shobe, J. L., & Carew, T. J. (2006). Intermediate-term processes in memory formation. *Curr.Opin.Neurobiol., 16*, 672-678.

Strausfeld, N. J., Hansen, L., Li, Y., Gomez, R. S., & Ito, K. (1998). Evolution, discovery, and interpretations of arthropod mushroom bodies. *Learn Mem., 5*, 11-37.

Suarez, L. D., Smal, L., & Delorenzi, A. (2010). Updating contextual information during consolidation as result of a new memory trace. *Neurobiol Learn Mem, 93*, 561-571.

Sullivan, J. M., & Beltz, B. S. (2001). Neural pathways connecting the deutocerebrum and lateral protocerebrum in the brains of decapod crustaceans. *J.Comp Neurol., 441*, 9-22.

Sullivan, J. M., & Beltz, B. S. (2005). Integration and segregation of inputs to higher-order neuropils of the crayfish brain. *J.Comp Neurol., 481*, 118-126.

Sztarker, J., Strausfeld, N. J., & Tomsic, D. (2005). Organization of optic lobes that support motion detection in a semiterrestrial crab. *J.Comp Neurol., 493*, 396-411.

Sztarker, J., & Tomsic, D. (2004). Binocular visual integration in the crustacean nervous system. *J.Comp Physiol A Neuroethol.Sens.Neural Behav.Physiol, 190*, 951-962.

Sztarker, J., & Tomsic, D. (2011). Brain modularity in arthropods: individual neurons that support "what" but not "where" memories. *J Neurosci, 31*, 8175-8180.

Tomsic, D., de Astrada, M. B., Sztarker, J., & Maldonado, H. (2009). Behavioral and neuronal attributes of short- and long-term habituation in the crab Chasmagnathus. *Neurobiol Learn Mem, 92*, 176-182.

Tomsic, D., Maldonado, H., & Rakitin, A. (1991). Morphine and GABA: effects on perception, escape response and long-term habituation to a danger stimulus in the crab Chasmagnathus. *Brain Res.Bull., 26*, 699-706.

Tomsic, D., Pedreira, M. E., Romano, A., Hermitte, G., & Maldonado, H. (1998). Context-US association as a determinat of long-term habituation in the crab Chasmagnathus. *Animal Leraning & Behavior, 26*, 196-209.

Tomsic, D., & Romano, A. (2013). A Multidisciplinary Approach to Learning and Memory in the Crab Neohelice (Chasmagnathus) granulata. *In* R. Menzel, & P. R. Benjamin (Eds.), *INVERTEBRATE LEARNING AND MEMORY* (pp. 337-355). AMSTERDAM: Academic Press.

Troncoso, J., & Maldonado, H. (2002). Two related forms of memory in the crab Chasmagnathus are differentially affected by NMDA receptor antagonists. *Pharmacol.Biochem.Behav., 72*, 251-265.

Tsien, J. Z., Huerta, P. T., & Tonegawa, S. (1996). The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. *Cell, 87*, 1327-1338.

Weinberger, N. M. (2006). Food for thought: honeybee foraging, memory, and acetylcholine. *Sci STKE, 2006*, pe23.

Wolff, G., Harzsch, S., Hansson, B. S., Brown, S., & Strausfeld, N. (2012). Neuronal organization of the hemiellipsoid body of the land hermit crab, Coenobita clypeatus: correspondence with the mushroom body ground pattern. *J Comp Neurol, 520*, 2824-2846.

Wolff, G. H., & Strausfeld, N. J. (2016). Genealogical correspondence of a forebrain centre implies an executive brain in the protostome-deuterostome bilaterian ancestor. *Philos Trans R Soc Lond B Biol Sci, 371*.

Wu, C. L., Xia, S., Fu, T. F., Wang, H., Chen, Y. H., Leong, D., Chiang, A. S., & Tully, T. (2007). Specific requirement of NMDA receptors for long-term memory consolidation in Drosophila ellipsoid body. *Nat Neurosci, 10*, 1578-1586.

Yagodin, S., Collin, C., Alkon, D. L., Sheppard, N. F., Jr., & Sattelle, D. B. (1999). Mapping membrane potential transients in crayfish (Procambarus clarkii) optic lobe neuropils with voltage-sensitive dyes. *J Neurophysiol, 81*, 334-344.

Figure 1. *Long-term memory and dissociation between memory expression and memory persistence.* **A and B left panels:** schemas of the experimental procedure and the successive sessions in each experiment. All animals were injected 15 min before training (Day 1) with saline solution (vehicle), scopolamine (0.1ug/g) or MK801 (1ug/g) when indicated. **A (right panel)** *Memory after a strong training protocol***:** *Day 1,* training session, escape response of the strongly trained group (TR, squares) for trials with the VDS. *Day 2 and 3,* escape response during tests. Memory expression is evidenced in the TR group as a lower escape response to the VDS in comparison to the untrained group (UN, diamonds) (UN-TR n= 35-34, independent t-test: **p<0.01, *p<0.05). **B right panel: Bi.** *Memory persistence after a weak training protocol. Day 1,* two weakly trained groups along trials of the training session. *Day 2-Reminder Session*: 24 h after training all animals were re-exposed for 5 min to the training context. At the end of this period, the TC+VDS groups received a single VDS trial (white symbols), memory expression was not disclosed because the weak training; the TC groups (dark symbols) finished the exposure without the VDS presentation. After the reminder session all animals were water deprived (w.d.). *Day 3, Testing Session*; memory expression was disclosed for the TC groups (**p<0.01, a priori planned comparisons test) but not for the TC+VDS groups (n=30-35 in each group)(replicated from Frenkel et al. 2005). **Bii.** Equivalent as Bi for strongly trained groups injected with scopolamine (SCP, 0.1 µg/g) pre training. Memory expression was disclosed at Day 3 among the TC groups (*p<0.05, a priori planned comparisons test, all groups: n=32) but not in the TC+VDS groups both at Day 2 and 3. **Biii.** Equivalent as Bi for a strong-trained group injected with MK801 (1 µg/g) pre training. No memory expression was disclosed for any of the groups (all groups: n=26). Abbreviations: UN: untrained; TR: trained; TC: training context; VDS: visual danger stimulus; w.d.: 2h water deprivation. Responses of untrained groups on day 1 are

not shown. The ordinates give mean +/- $CI(95\%)_{Loftus\&Masson}$ of the escape response (log2 transformed) elicited by VDS.

Figure 2. *Crab preparation for Calcium imaging*. **A.** Crab held in the *ad hoc* clamp and chelae restrained with a rubber band (arrowhead). Note the eyestalks (arrow) resting down as a sign of anesthesia produced by the cold water. **B.** Left eyestalk fixed in position. **C.** Lateral protocerebrum (LP) accessed through a window cut in the cuticle. The connective tissue and muscles were removed and the neuroepithelium was cut and attached to the edges of the cuticle. Around the eyestalk, a container for Ringer's solution was build up using dental cement. **D.** Montage schema showing the neuropils in the eyestalk and the point where the CalciumGreen 1- dextran crystal was inserted (★). Shaded rectangle and detail illustrate the calcium imaging recorded area. **E.** Confocal obtained images of fixed tissue where an example of the stained neurons can be observed. The picture corresponds to a stack of sections that include the registered area (approximately shown by dashed line) corresponding to the medulla terminalis (MT). The arrow points toward the place where the microelectrode with the dye was stabbed. **F.** Stack at the level of the hemiellipsoid body neuropil, located at least 100 µm below the focus plane of the registered *medulla terminalis* (E-F bottom: depth (µm) of confocal images). N: nerve; LP: lateral protocerebrum; MT: *medulla terminalis*; Lb: *lobula*; sg: sinus gland; HB: hemiellipsoid body. D, dorsal; A, anterior; L, lateral; M, medial are taken with the eyestalk in upright posture in the intact animal (Sztarker, Strausfeld, and Tomsic, 2005).

Figure 3. *Detail of the stimuli trials and the training protocol during calcium imaging experiments.* **A.** *Visual danger stimulus (VDS).* Left, image showing the crab positioned in the microscope and the position of the VDS. Note the upright posture of the contralateral stimulated eye (inset). Right, timeline showing the two cycles of a VDS trial. **B.** *Mechanical stimulus (MS).* Left, image showing

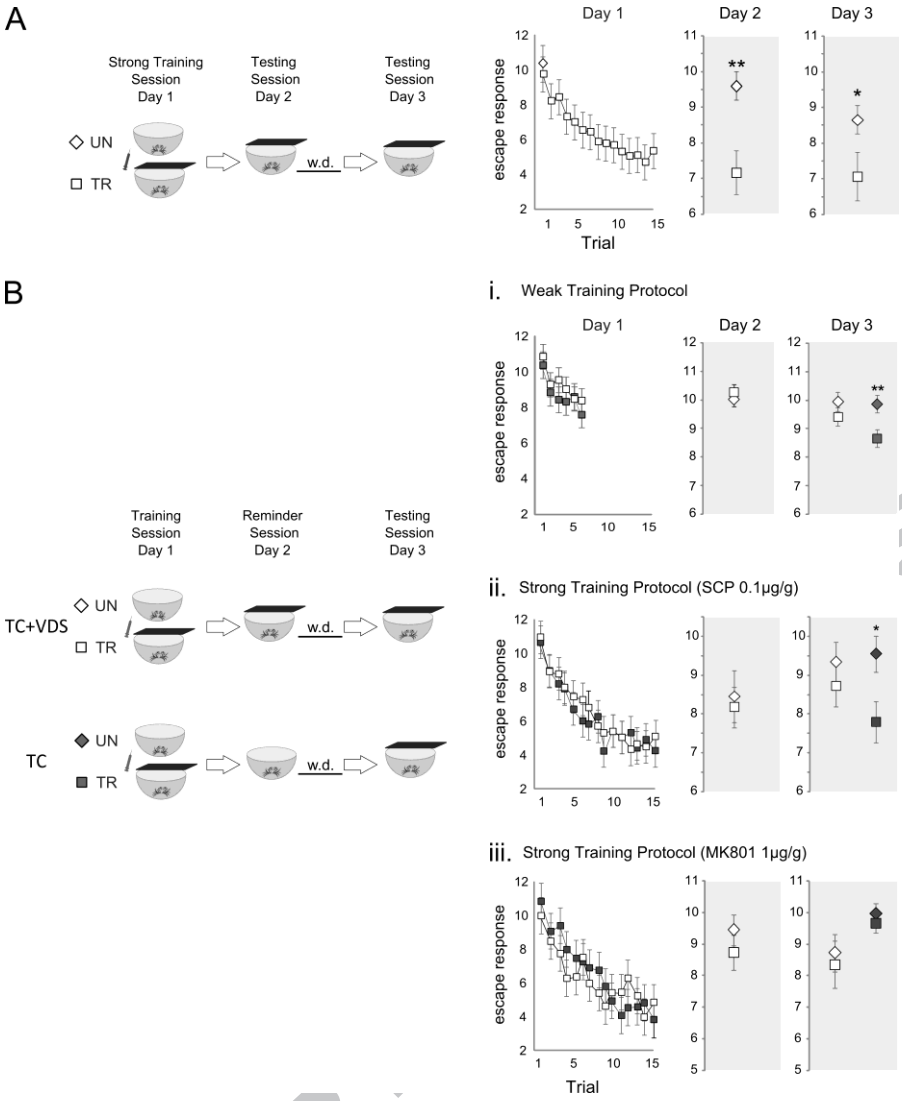
the crab positioned in the microscope and the air injector (MS). Right, timeline showing pulses of a MS trial. **C.** Schematic timeline illustrating the mechanical and visual stimulation during training and imaging experiments.

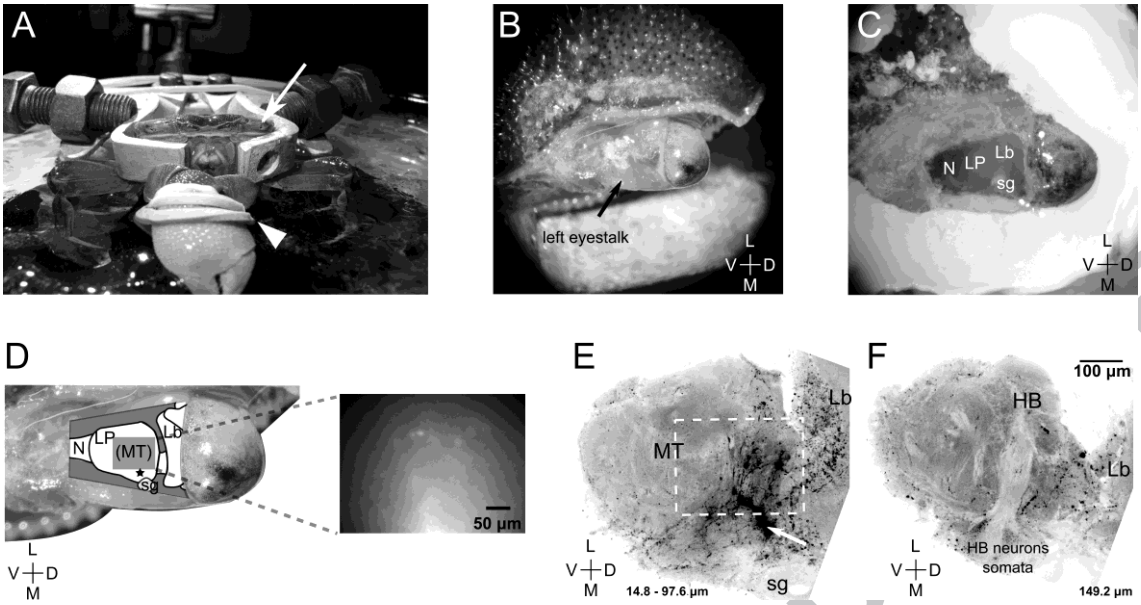
Figure 4. *Calcium responses in the medulla terminalis during strong training and test and the effect of NMDA receptors blockade.* The figure includes an example of a strong trained crab injected with vehicle (A, B) and a strong trained crab injected with MK801 1 µg/g (C, D). **A, C.** False color-coded images representing the summation of %deltaF/F for each pixel during stimuli cycles, first row: 1st cycle, second row: 2nd cycle. The black line superimposed in the false-color pictures delineates the AOI for those animals. D, dorsal; A, anterior; L, lateral; M, medial. Scale bar, 50µm. **B, D.** Calcium dynamics during trials. The traces represent the temporal detail of the mean %deltaF/F in the AOI during the recording time. The shaded area below the traces corresponds to the summation of %deltaF/F, considered for analyses. Black triangles or rectangles along the X-axes indicate the time of stimulation, VDS or MS respectively. Abbreviations: VDS, visual danger stimulus; MS, mechanical stimulus.

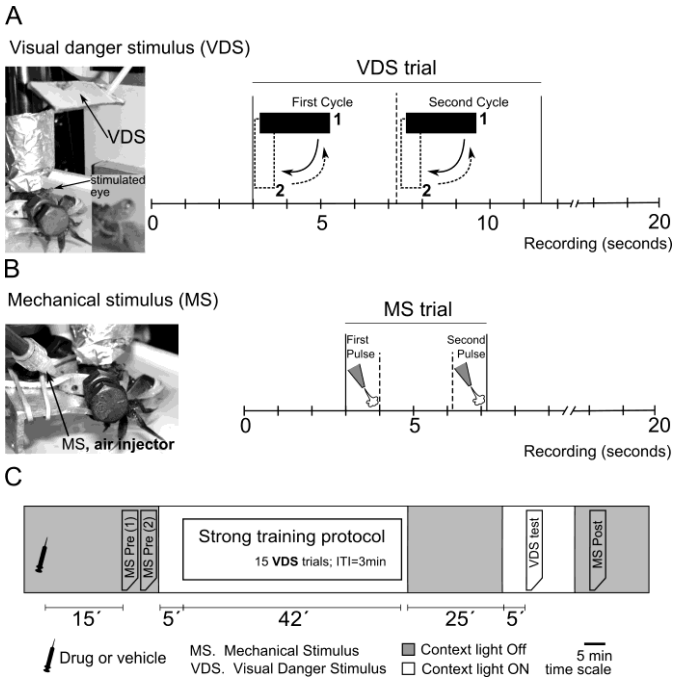
Figure 5. *Changes in calcium responses in the medulla terminalis after different training protocols and pharmacological treatments.* In all cases bars represent the mean +/- $CI(95\%)_{Loftus\&Masson}$ of the summation of %deltaF/F elicited by the first (**i**) or the second cycle (**ii**) of the VDS; only trials 1, 6, 15 and test trial are shown. **(iii**) Summation of %deltaF/F elicited by the first and the second pulse of the MS; PRE refers to the first MS before training and POST to the MS after test with the VDS. **A.** Strong training protocol, 15 VDS trials + 1 test trial, (n=6). **B.** Untrained group, 1 VDS trial + 1 test trial (n=5). **C.** Weak training, 6 trials + 1 test trial (n=6). **D.** Strong training protocol, scopolamine 0.1μg/g (i,ii: n=5 ,iii: n=4). **E** Strong training protocol, scopolamine 5 μg/g (n=6). **F.** Strong training protocol, MK-801 1 μg/g (i,ii: n=6 ;iii: n=5). Post hoc LSD test, *** $p<0.001$, **

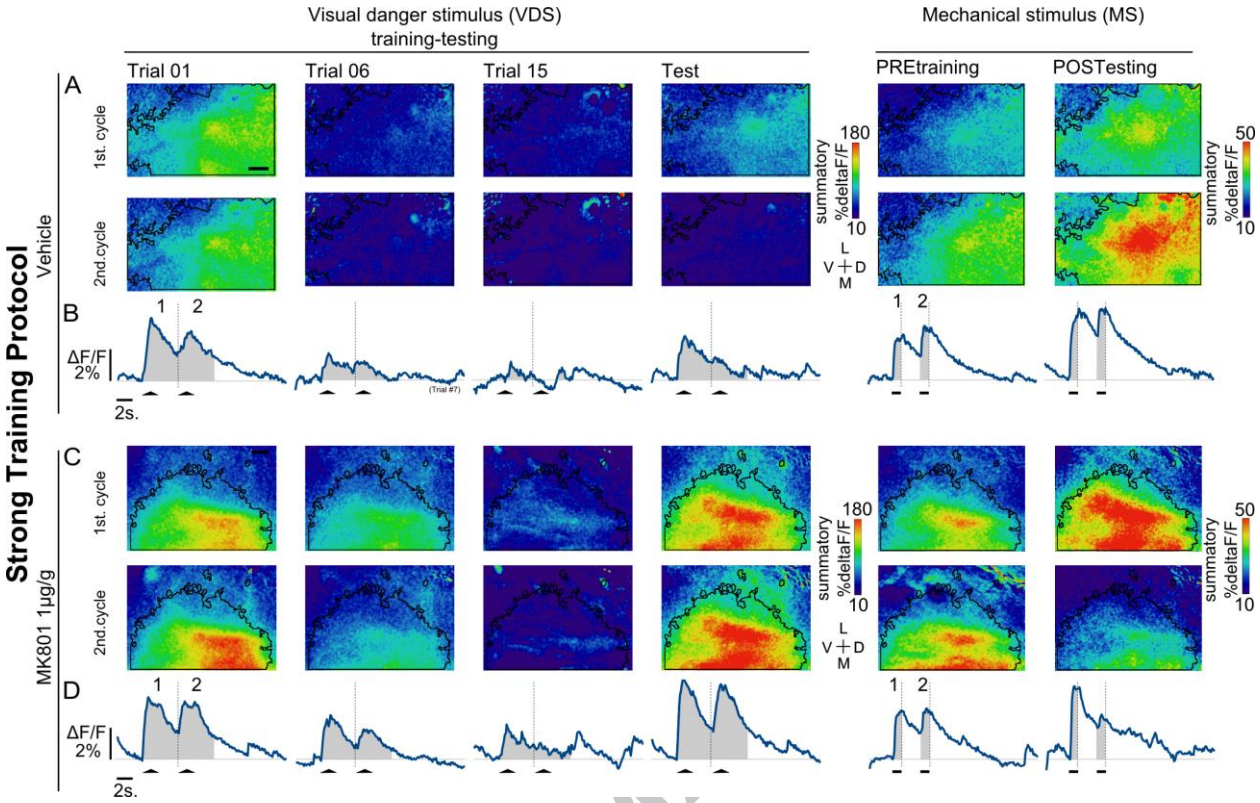
p<0.01,*p<0.05; significant differences with its corresponding first VDS trial or with the PRE trial MS.

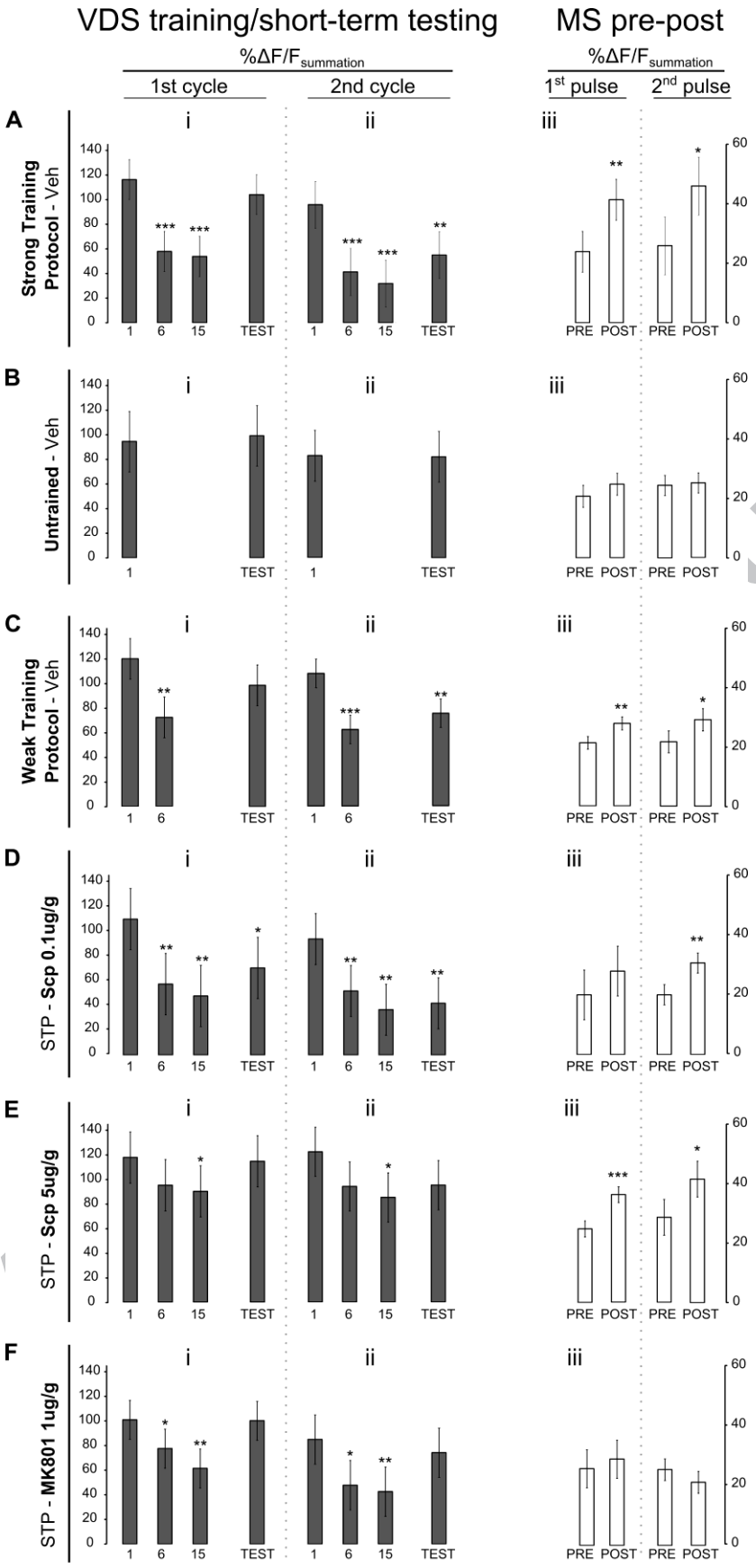
Table 1. *Summary of results showing changes in Ca2+ responses, behavioral short and long-term memory expression and long-term memory persistence.* **Memory expression**: √ when memory is evidenced by a lower escape response; X when memory is not evident in the animal´s behavior. **LTM persistence:** √ indicates whether there is a memory that is behaviorally expressed or can be modulated during reconsolidation; X: when memory is not evident even after memory enhancement treatment. **Medulla terminalis´s neural plasticity:** changes observed in calcium responses elicited by the VDS (down or up arrows correspond to decreased or increased responses toward the stimulus). a: Figure 1B, (Frenkel et al., 2005a); b: Figure 1B, (Beron de Astrada and Maldonado, 1999; Caffaro et al., 2012); c: Figure 1B, (Caffaro et al., 2012); d: (Caffaro et al., 2012); e: Figure 1B, (Troncoso and Maldonado, 2002). Abbreviations: STP, strong training protocol; MS, mechanical stimulus; VDS, visual danger stimulus; STM: short-term memory; LTM: long-term memory.











	Memory Expression		LTM Persistence	*Medulla Terminalis* Neural Plasticity	
	STM	LTM		VDS	MS
Strong Training Protocol	✓	✓	✓	↓	↑
Weak Training Protocol	✓	X	✓ (a)	↓	↑
STP-Scopolamine $_{0.1\mu g/g}$	✓	X (b)	✓ (c)	↓	↑
STP-Scopolamine $_{5\mu g/g}$	✓	X	X (d)	=	↑
STP- MK-801 $_{1\mu g/g}$	✓	X (e)	X (e)	=	=
Untrained	–	–	–	=	=

Title: Neural correlates of expression-independent memories in the crab Neohelice.

Authors: Francisco Javier Maza, Fernando Locatelli and Alejandro Delorenzi.

Highlights

- Medulla terminalis neuropil activity reflects components of LTM traces in Crustaceans.
- Changes in neural activity correlate with memory persistence.
- Changes do not correlate with the probability of LTM to be expressed.
- Memory persistence and expression might involve separate components of traces.
- Memory retrieval and memory expression might not be interchangeable concepts.