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Long-term habituation (LTH) in the crab *Chasmagnathus*: a model for behavioral and mechanistic studies of memory

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Abstract

A decade of studies on long-term habituation (LTH) in the crab *Chasmagnathus* is reviewed. Upon sudden presentation of a passing object overhead, the crab reacts with an escape response that habituates promptly and for at least five days. LTH proved to be an instance of associative memory and showed context, stimulus frequency and circadian phase specificity. A strong training protocol (STP) (≥15 trials, intertrial interval (ITI) of 171 s) invariably yielded LTH, while a weak training protocol (WTP) (≤10 trials, ITI = 171 s) invariably failed. STP was used with a presumably amnestic agent and WTP with a presumably hypermnestic agent. Remarkably, systemic administration of low doses was effective, which is likely to be due to the lack of an endothelial blood-brain barrier. LTH was blocked by inhibitors of protein and RNA synthesis, enhanced by protein kinase A (PKA) activators and reduced by PKA inhibitors, facilitated by angiotensin II and IV and disrupted by saralasin. The presence of angiotensins and related compounds in the crab brain was demonstrated. Diverse results suggest that LTH includes two components: an initial memory produced by spaced training and mainly expressed at an initial phase of testing, and a retraining memory produced by massed training and expressed at a later phase of testing (retraining). The initial memory would be associative, context specific and sensitive to cycloheximide, while the retraining memory would be nonassociative, context independent and insensitive to cycloheximide.

Key words
- Memory
- Massed training
- Spaced training
- Cycloheximide
- PKA
- Angiotensin

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Simple and basic models to study memory

Simple-system approaches using invertebrate species have led to considerable progress in our understanding of the mechanisms underlying learning and memory (e.g., the mollusks *Aplysia* (1,2) and *Hermissenda* (3,4), the fruit fly *Drosophila* (5), and the honey bee *Apis* (6)). A virtue usually attributed to invertebrates is that they are simple models though the use of this term has been highly controversial. Indeed, the CNS of invertebrates cannot be considered simple from the perspective of a neural network researcher since the amount of neurons involved in a current mnemonic process is assumed to be far larger than that required to obtain a satis-

factory description of the underlying circuit. For example, the CNS of *Aplysia* contains approximately 20,000 neurons and hundreds, if not thousands, of these neurons are probably active during classical conditioning of *Aplysia*'s gill- and siphon-withdrawal reflexes (7). However, the level of complexity of the invertebrates used as memory systems to date is very much lower than that of vertebrates, so that the basic mechanisms that subserve memory become methodologically much more accessible, and therefore the term basic system is more appropriate than that of simple system.

A current assumption in studies with a basic system of memory is that conclusions obtained with such research could be extended to other animals, including humans. This possibility is based on the fact that animals of very different species share many elementary anatomical and functional structures. Many neurotransmitters, key molecules and entire signal transduction pathways have emerged early in evolution and have been largely conserved. In other words, the assumption is that the basic core of memory is the same from annelids to humans and that the main differences between species are given by the emergent properties that arise as a consequence of increasing complexity.

Thirty years ago a fascinating research was started aimed at simplifying the process of memory to its most essential components and since then outstanding progress has been made in identifying key molecules associated with long-term synaptic plasticity (e.g., 8,9). However, it is still necessary to determine whether these findings are really pertinent to long-term memory. Consequently, it has been argued that the relevance to memory of these results should be tested in a behaving animal (an appropriate basic model), thus bringing the search for the cellular and molecular basis of memory back to the behaviors it was designed to explain (10).

With this theoretical reference framework we are presenting in this review the most salient results obtained over more than ten years using a basic model of memory, namely the long-term habituation (LTH) of the crab *Chasmagnathus granulatus*.

The protagonist

The semiterrestrial crab *Chasmagnathus* is found along the coast of South Brazil, Uruguay and Argentina, occupying mud flats of the mesolittoral and supralittoral zones of fresh-seawater transition. For our study, adult male crabs of similar size were easily captured throughout the year from the rias (narrow coastal inlets) of San Clemente del Tuyú, Argentina, and transported to the laboratory. The animals were kept in plastic tanks filled to 2 cm depth with artificial sea water and fed rabbit pellets every 3 days. After feeding the water was changed and the animals appeared to be healthy up to at least 15 days after arrival.

An automatic device to study long-term habituation in crabs

Upon sudden presentation of a rectangular screen passing overhead, the crab responds with a running reaction in an attempt to escape. The flight reaction declines over various trials and the decrement persists for at least five days (11,12).

To study this process, a wholly automated device that allows the experimenter to work with 40 animals simultaneously was used. The apparatus is described in detail elsewhere (13). Briefly, the experimental unit is the actometer (Figure 1a), consisting of a bowl-shaped plastic container (C) with a steep concave wall and a flat central circle, covered to a depth of 0.5 cm with water. The crab was placed in the container which was suspended by three strings from an upper wooden framework and was illuminated with a 10-W lamp placed 30 cm above the animal. An opaque rectangular screen (R) was moved horizontally by a motor (M) over the animal

and across the upper surface of the framework, thus provoking a running response of the crab and consequently container oscillations. A stylus was centrally cemented to the bottom of the container and connected to a piezoelectric transducer (P). Container oscillations induced electrical signals proportional to the velocity of the oscillations through the transducer. These signals were amplified, integrated during the recording time (9 s) and translated into numerical units ranging from zero to 1530, before being processed with a computer. The experimental room had 40 actometers, separated from each other by partitions. A computer was employed to program trial sequences, trial duration and intertrial intervals, as well as to monitor experimental events.

During an experiment, crabs were usually distributed into pairs of groups, each pair consisting of a trained group (TR) and a control group (CT). The experimental procedure for a TR could be summarized as follows. A stimulation session consisted of a fixed number of trials given with an intertrial interval (ITI) of 171 s and preceded by 15 min of adaptation to the actometer. Each trial lasted 9 s and consisted of passing the screen four times over the actometer and recording the activity of the crab throughout the trial time. Two sessions per experiment were run, i.e., the training session (5, 10, 15 or 30 trials) and the testing session (6 trials), separated by 24 or more hours. During the intersession interval, crabs were individually lodged in rest containers covered with water to a depth of 0.5 cm and kept inside dimly lit drawers.

The experimental protocol for a CT was the same as that of TR in all respects except that animals of this group stayed in the actometers during the entire training session without being trained, i.e., without being presented to the habituating stimulus. Retention for long-term habituation is operationally defined as the significant difference between CT and TR in a testing session.

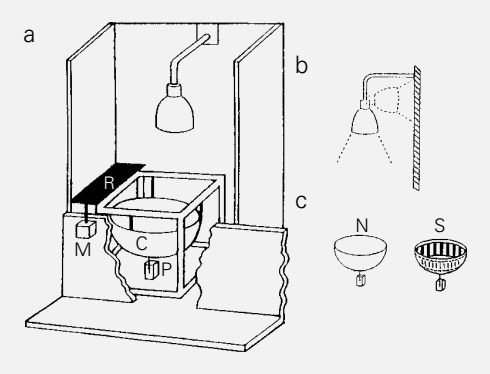

Figure 1 - *a*, The actometer, one of the 40 units of the apparatus. C: Plastic container; R: rectangular screen; M: motor; P: piezoelectric transducer. *b*, Training-to-testing change in context experiment: the lamp that illuminates the actometer container from above is rotated 90 degrees and directed towards the back wall of the actometer. *c*, Idem: a ring with black and white vertical stripes (S) is located inside the usual actometer container (N).

Strong *vs* weak training protocol

A trained group underwent 15 or more training trials when a strong training protocol was used (Figure 2). The sharp fall in response shown by TR during the training session corresponds to the so-called short-term habituation (STH) (Figure 2, left panels). After the 24-h intersession interval, most of the testing trials showed a significant difference between TR and CT, i.e., the strong protocol yielded a robust LTH (Figure 2, middle upper panel). A similar pattern of results was invariably obtained in all experiments in our laboratory provided that 20 or more crabs per group were used, though very often the most constant difference was found during the first two testing trials. In contrast, ten or fewer training trials invariably failed to induce LTH (Figure 2, middle lower panel).

The advantage of having such contrasting and reliable results with two protocols that hardly differ in the amount of training is apparent mainly for interventive experiments. Thus, a strong training protocol is used when an agent presumably has an amnestic effect, while a weak training protocol is adopted when it is assumed to have a hypermnestic (= facilitatory) effect (Figure 2, right panels).

Behavioral experiments

The adaptive value of LTH

Since the very beginning of our work, the

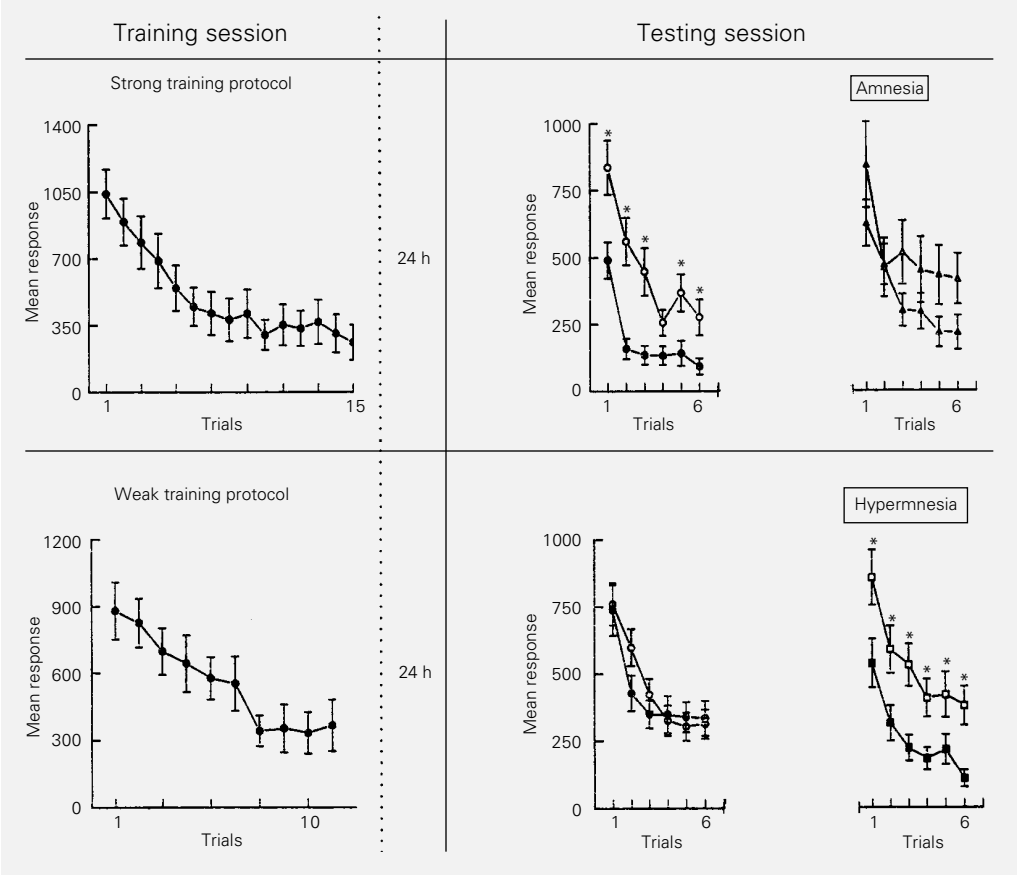

Figure 2 - Strong *vs* weak training protocol. Ordinate: mean score per trial ± SEM. *Left panels*: *top*, the trained group receives 15 trials (ITI = 171 s) (the control group stays in the actometer without training); *bottom*, idem but the trained group receives 10 trials. *Middle panels*: testing of untreated groups. *Right panels*: *top*, testing of groups treated with an amnestic agent; *bottom*, idem with a hypermnestic agent. Higher curves correspond to control groups and lower curves to trained groups. *P<0.01 compared to control in each trial. Intersession interval: 24 h.

finding that a robust LTH was built up by a single session of 15 training trials (= 45 min of training) and was exhibited after a 5-day intersession interval was considered a rather surprising result. In fact, the fright response of the crab induced by an object moving overhead suggests that this visual stimulus stands for an impeding threat, so that a persistent reduction of a defensive response would seem to be nonadaptive for the species. Therefore, an extensive study was performed to ascertain the possible biological meaning of the robust LTH (14). For this, the capacity for yielding LTH in *Chasmagnathus* and in *Pachygrapsus*, two closely related grapsid species that diverge widely in ecology, was compared using the same experimental set up and design. Clear-cut results were obtained: *Chasmagnathus* showed a far greater capacity for LTH than *Pachygrapsus* and this difference appears to correlate well with ecological disparity. The former lives immersed in an environment featuring wind oscillations of cord grasses while the latter inhabits a bare biotope of rocky promontories, so that the probability that a passing object signals an actual predator is lower for *Chasmagnathus* and consequently larger its capacity for acquiring LTH.

LTH includes a component of associative memory

The decrease in the crab reactivity within the training session (STH) was shown to meet most of the parametric criteria of habituation (15). However, four series of results strongly suggest that LTH can be appropriately categorized as an instance of associative memory, thus supporting Wagner's associative theory of habituation (16,17), i.e., STH would depend solely upon the it-

erative presentation of the phasic stimulus (= the passing screen overhead, = the danger stimulus) while LTH would result from an association between the phasic stimulus and the context in which it is presented (17). Specifically, results of the four series were as follows. 1) *Chasmagnathus* LTH was impaired when the context was changed between sessions (12,18; Tomsic D, Pedreira ME, Romano A and Maldonado H, unpublished data), either by reorienting the lamp over the actometer (Figure 1b) or by placing a ring with black and white vertical strips inside the actometer (Figure 1c); 2) pre-training exposure to the contextual cues in the absence of the phasic stimulus, namely the classical conditioning procedure of latent inhibition (19), reduced LTH (Tomsic D, Pedreira ME, Romano A and Maldonado H, unpublished data); 3) post-training exposure to the context without phasic stimulation, namely the classical conditioning procedure of extinction, produced a decrease in LTH (Tomsic D, Pedreira ME, Romano A and Maldonado H, unpublished data); 4) LTH tended to vanish with an increasing reduction of the rest interval between trials (Pedreira ME, Romano A, Tomsic D, Lozada M and Maldonado H, unpublished data), an outcome in keeping with a large body of evidence from experiments of classical conditioning (1,20) but at odds with the current view of the nonassociative theory of habituation (21).

A closer analysis of the foregoing results shows that the LTH impairment produced by context shift as well as by pre-training or post-training exposure was expressed only during the initial phase of testing (first trial or first 2-trial block of testing) but not during the final phase (the last four or five testing trials). This disparity suggests that LTH may include both an associative and a nonassociative component: the former, evoked by the initial exposure to context, subserves retention during the first testing trial, while the latter, evoked by the first trial at testing, is expressed during the subsequent test trials. Such interpretation is similar to that proposed by the dual-process habituation model (22,23).

Chasmagnathus learns the phase of the day when LTH is acquired

The response level of *Chasmagnathus* to the danger stimulus during the dark phase (defined for these experiments as a time interval between 22:30 and 1:30) was higher than that observed during the light phase (between 10:30 and 13:30) and, coincidentally, the level of locomotor activity in the actometer showed a circadian rhythm with a peak during the subjective night. However, LTH was impaired in crabs tested at a time of day that differed from that of their original training, regardless of the daily phase of the training, testing session or the extent of the intersession interval, i.e., a robust retention was found 24, 48 or 72 h after training but not after 12 or 36 h (24).

The foregoing finding is reminiscent of the so-called state-dependent retention, an effect commonly observed with drug-induced states (25) but also induced nonpharmacologically (26,27). On an interpretative level, it appears that the retention of the habituated response depends on the congruence between the internal state of the crab at the time of training and testing, a notion consistent with Spear's view of memory retrieval (28,29). This type of interpretation assumes that there is an oscillation of the internal state, namely a differential set of interoceptive cues across time of day, and, on the other hand, that memory for the internal state at training time is stored together with the memory of the context-stimulus association. Briefly, LTH is circadian-phase specific.

Chasmagnathus learns the rest time interval between trials during LTH acquisition

Recent results (Pedreira ME, Romano A,

Tomsic D, Lozada M and Maldonado H, unpublished data) have confirmed and extended previous results (30) showing that LTH retention is impaired when different intertrial intervals (ITI) are used at training and testing. Thus, when a strong training protocol is used with either 45- or 135-s ITI instead of the usual one of 171 s, an equally robust LTH is obtained provided that 45- or 135-s ITI were included at testing. In contrast, LTH is impaired when ITI shifts from 45 to 135 s or vice versa. This phenomenon is found with different combinations of ITI and when a relation of 3.0-fold between intervals is respected.

Therefore, LTH is frequency specific, a result that, along with those showing context and circadian-phase specificity, contrasts with the current view of habituation as an extremely simple process. Indeed, several authors share the idea that habituation arises solely from changes that are intrinsic to a reflex pathway and that all the processes could be reduced to acquire a single item of memory, namely that for the iterated stimulus. More explicitly, Hawkins and Kandel (31) proposed the hypothesis that all the instances of complex memories could be explained by combining units of exceedingly simple memories (irreducible modules of memory) such as habituation and sensitization.

Massed and spaced training build up different memory components of LTH

Several results from our laboratory have suggested the possibility that the impact of LTH on the first trial or on the first 2-trial block of the testing session (initial testing phase) was different from the impact on the following block of trials (retraining phase), suggesting that different mechanisms may be subserving memory at different testing phases. The following results support this distinction. 1) When animals underwent a strong training protocol, the largest difference between the CT and TR groups generally occurred during the initial part of the testing curve, then decreasing over trials (14). It is worth emphasizing that an opposite picture of results was obtained for *Pachygrapsus*: CT and TR were invariably indistinguishable during the initial phase of testing but significant differences emerged over trials. 2) When animals underwent a weak training or were submitted to a strong training protocol followed by the injection of an amnestic agent, any CT-TR difference during the initial phase of testing vanished (e.g., 12,18,32), though sporadic CT-TR differences appeared in a few trials of the retraining phase. 3) When a weak training was carried out and a facilitatory agent was administered after training, a CT-TR difference was disclosed during the initial testing phase but not necessarily during retraining (32). 4) A context shift as well as pre- or post-training exposure to the actometer in the absence of the phasic stimulus impaired LTH during the initial phase of testing but not at retraining, suggesting that LTH may be composed of an associative and a nonassociative component, respectively (Tomsic D, Pedreira ME, Romano A and Maldonado H, unpublished data).

A recent study (Pedreira ME, Romano A, Tomsic D, Lozada M and Maldonado H, unpublished data) was carried out to determine how the magnitude of these two putative memory components of LTH are influenced by the training stimulus frequency, i.e., the length of the training ITI. For this purpose, the experimental data were analyzed separately for the initial testing phase (confined in this case to the first testing trial) and retraining (the block of the last five testing trials). Four examples from a long series of experiments are illustrated in Figure 3. Figure 3 A, B and C shows the testing performances of groups that received 15 training trials and stayed in the actometers for 45 min, but one group was given a 171-s ITI (Figure 3A), another group had a 27-s ITI

(Figure 3B) and a third group had no rest interval between trials (Figure 3C). With a training ITI longer than 27 s a significant difference was always found both during the initial testing phase and at retraining; with the 27-s training ITI (a boundary case), LTH was expressed during the initial testing phase but not at retraining, and with massive training no retention was expressed during any testing phase. However, when the number of trials of the massive training was increased up to 300 trials (i.e., 45 min of continuous stimulation) (Figure 3D), LTH was expressed at retraining but not during the initial phase of testing. The main conclusion from this behavioral analysis is that one memory component of LTH is only produced by spaced training though expressed both during the initial testing phase and at retraining, and consequently termed initial memory, and another one produced by massed training and expressed only at retraining, and consequently termed retraining memory.

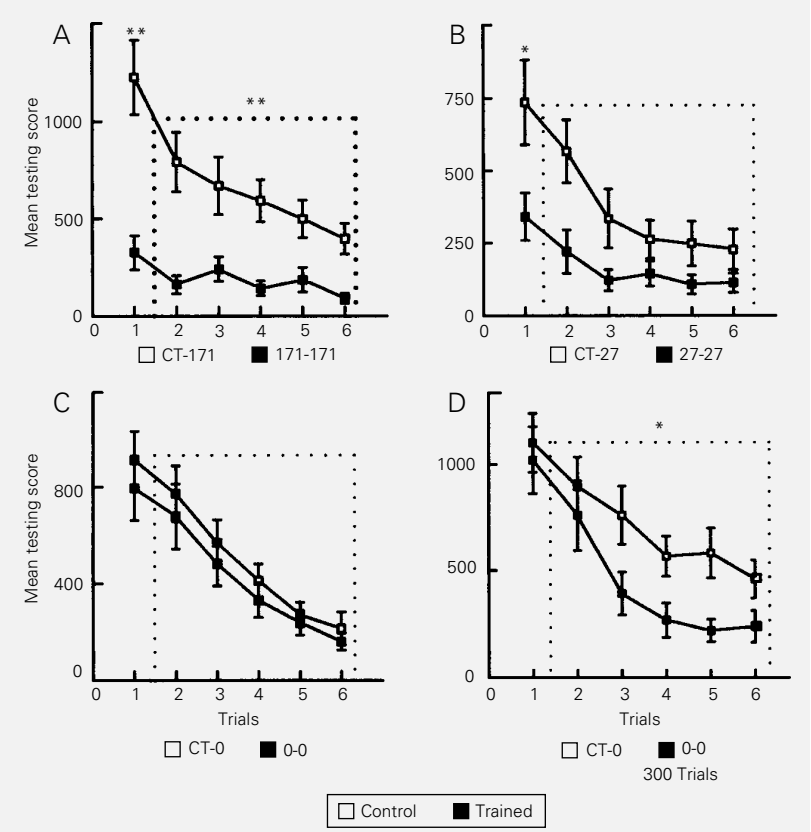

Figure 3 - Massed and spaced training. Testing performance (6 trials). Ordinate: mean score per trial ± SEM. CT, Control group; TR, trained group. *A*, TR received 15 training trials, ITI = 171 s. *B*, Idem but ITI = 27 s. *C*, Idem but ITI = 0 s. *D*, TR had received 300 trials, ITI = 0 s. *P<0.01 between TR and CT at first testing trial (initial phase) or at retraining (dotted box); **idem but P<0.005. Intersession interval: 24 h.

Pharmacological and biochemical experiments

The lack of an endothelial blood-brain barrier in *Chasmagnathus* enhances the action of compounds given by systemic administration

The injection procedure in pharmacological experiments with *Chasmagnathus* was as follows. Fifty µl of vehicle or drug solution was injected through the right side of the dorsal cephalothoracic-abdominal membrane by means of a syringe fitted with a sleeve to control depth of penetration to 4 mm, thus ensuring that the injected solution was released roughly at the center of the pericardial sac (e.g., 32-34). A remarkable feature shared by all these experiments was that the effective drug doses given by systemic administration were manifestly low, i.e., equivalent to or even lower than doses administered by intracranial injection in vertebrates, e.g., cycloheximide (CYX) (12), actinomycin-D (ATY) (18), angiotensin II (35), enkephalin (33), serotonin (36). The relative simplicity of brain organization and the lack of an endothelial blood-brain barrier in crabs (37), together with the fact that blood is distributed throughout an extensive capillary system in various neuropil areas of the brain (38,39), may explain the low threshold for drug action. The possibility of working with such low doses by systemic injection makes this a suitable approach to study the mechanistic aspects of mnemonic processes.

LTH is blocked by inhibitors of protein and RNA synthesis

A first step in a study aimed at elucidat-

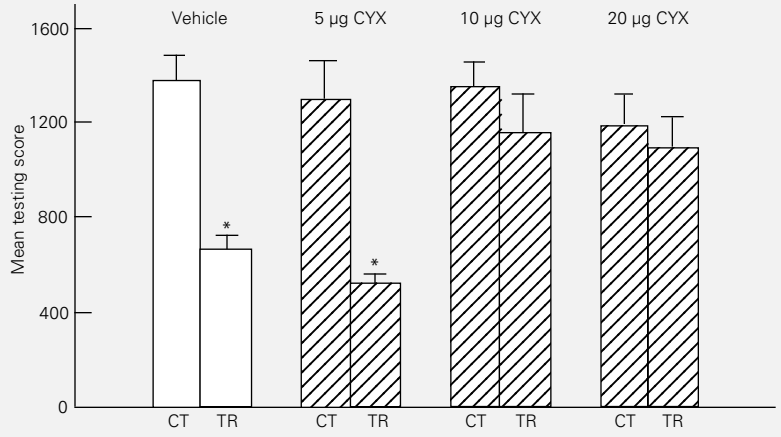

Figure 4 - Cycloheximide (CYX) impairs LTH. Testing performance (6 trials). Ordinate: mean score per trial ± SEM. Injections after a strong training protocol (30 trials). Open bars: injection with vehicle; striped bars: idem with CYX. CT, Control group; TR, trained group. *P<0.05 compared to CT injected with vehicle. Intersession interval: 72 h.

ing the mechanisms that subserve *Chasmagnathus* LTH was to inquire to what extent this mnemonic process is protein synthesis dependent. Thus, a series of experiments was performed aimed at testing the effect of CYX and ATY on acquisition and retention of LTH (12,18).

An injection of 10 µg of CYX inhibited incorporation of [^{14}C]-amino acids into brain protein by 88% for 2 h and 20 µg inhibited it by 92%, while 0.6 µg of ATY inhibited incorporation by 59.7% for 1 h, but no inhibition was found at 24 h postinjection in any case. To test the effect of blocking protein synthesis on LTH, the strong training protocol (15 or 30 trials separated by 171 s) was used and the impact was assessed considering only the performance during the first testing trial or the first 2-trial block, i.e., during the so-called initial phase of testing. Both CYX (10-20 µg) and ATY (0.6 µg) administered immediately after training impaired LTH tested at 24 h. The amnestic effect of CYX was also shown at 72 or 120 h of intersession interval when 10-20 µg CYX but not 5 µg were injected (an example of these experiments is illustrated in Figure 4). The amnestic window of these drugs proved to be very narrow since no memory impairment was found with doses equal to or less than 5 µg CYX or 0.5 µg ATY whereas overt symptoms of sickness appeared 24 h after injection with doses hardly higher than 20 µg CYX or 0.7 µg ATY. Concerning the time window of the CYX effect, experiments carried out with 20 µg CYX showed amnesia when the drug was injected 2 h after training but not after 6 h. To address the possibility that the effect of CYX cited above might be an instance of state dependence (e.g., 40,41), an experiment was conducted in which vehicle or drugs were injected pre-training and pre-testing. LTH impairment was only shown in those combinations that included an injection of CYX before training.

In other experiments two groups of crabs received vehicle (control group) or 20 µg CYX (treated group) before training, and after 30 min of adaptation were submitted to a strong training of 30 trials. No difference between the training curves obtained for the two groups was found, suggesting that the preinjection of CYX has no effect on STH.

Apart from their amnestic effect, CYX and ATY only have an inhibitory effect on protein synthesis in common: CYX acts directly on the translation step while ATY acts indirectly by blocking total RNA synthesis. Therefore, the present results support the view that *Chasmagnathus* LTH, unlike STH, requires *de novo* protein synthesis.

A current observation in experiments with CYX was that the amnestic effect was conspicuous during the initial phase of testing but poor or absent at retraining. This differential effect of CYX is in keeping with the differential effect of context shift and stimulus frequency previously discussed in this review. Therefore, it is tempting to draw a comprehensive conclusion, namely that the initial memory is context specific and sensitive to cycloheximide, while the retraining memory is independent of the context and insensitive to cycloheximide. Obviously, such dual-process habituation model requires further research to obtain stronger empirical underpinnings. Specifically, experiments aimed at testing the ef-

fect of cycloheximide and context shift on either LTH after massed training or after spaced training are necessary. Incidentally, it is worth emphasizing the similarity between the hypothesis proposed for *Chasmagnathus* and that advanced for olfactory learning in *Drosophila* (5), i.e., the initial and retraining LTH of *Chasmagnathus* parallels long-term memory and anesthesia-resistant memory of *Drosophila*, respectively.

cAMP-dependent protein kinase A (PKA) mediates *Chasmagnathus* LTH

Initially, it was reported that systemic administration of the cAMP membrane permeable analog 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) plus the phosphodiesterase inhibitor isobutyl methylxanthine (IBMX) induced LTH when injected before or immediately after a 5-trial training session (42). Although such a result supports the view that an increase in cAMP level is one of the key steps in the consolidation of crab LTH, it is not possible to definitely conclude that cAMP-dependent PKA mediates the mnemonic process. Actually, it is known that CPT-cAMP also activates cGMP-dependent protein kinase (43) and that either IBMX or CPT-cAMP increases cGMP levels (44) so that an explanation in terms of mechanisms other than activation of PKA may account for the facilitatory effect of CPT-cAMP + IBMX. Therefore, further experiments (45) were aimed at studying the effect of the PKA activator Sp-5,6-DCl-cBIMPS (ACT) and the inhibitor Rp-8-Cl-cAMPS (INH) on retention level of the habituated response, tested 24 h after a weak training protocol (5 trials) or after a strong training protocol (15 or 30 trials), respectively. ACT and INH are membrane-permeant, metabolically stable analogs of cAMP whose action is highly specific for PKA, both interacting with the regulatory unit of this kinase (43,46). If PKA activation were a necessary step for memory storage in this learning paradigm, a facilitatory effect of ACT on retention would be expected to occur after the weak protocol and an amnestic effect of INH would be expected to occur after the strong protocol. The results were conclusive. A CT-TR difference was shown mainly at the initial phase of testing when crabs were injected immediately before or after a weak training. In contrast, a strong training failed to yield LTH when INH was injected pre- or post-training. Figure 5 shows an example of these results.

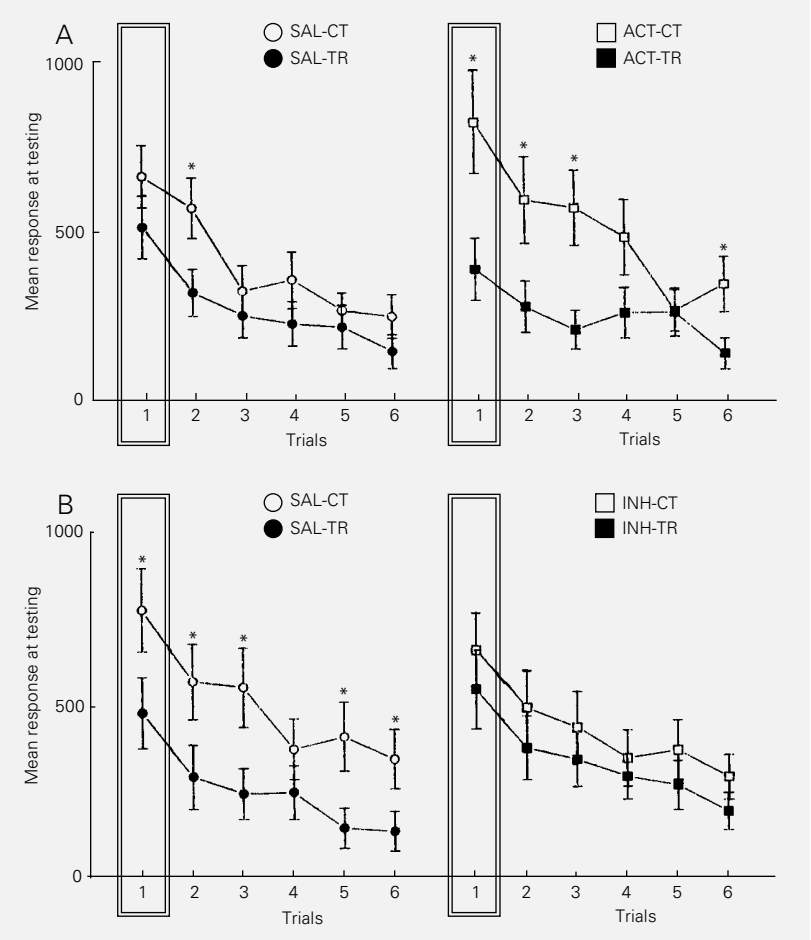

Figure 5 - LTH is mediated by PKA. Testing performance (6 trials). Ordinate: mean score per trial ± SEM. *A*, Effect of 75 µM Sp-5,6-DCl-cBIMPS (ACT) pre-training injection on LTH. Weak training protocol (5 trials). SAL-CT, Control group injected with saline; SAL-TR, trained group injected with saline; ACT-CT, control injected with ACT; ACT-TR, trained group injected with ACT. *B*, Effect of 25 µM Rp-8-Cl-cAMPS (INH) pre-training injection on LTH. Strong training protocol (15 trials). SAL-CT and SAL-TR as in *A*; INH-CT, control injected with INH; INH-TR, trained group injected with INH. *P<0.05 compared to control. Intersession interval: 24 h. A box highlights the first testing trial.

In all the experiments with pre-training injection, no difference between the saline

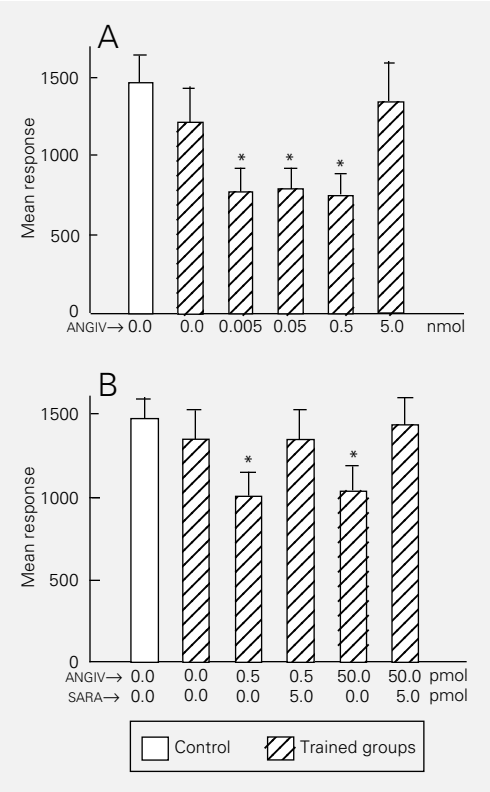

Figure 6 - Angiotensin IV (ANGIV) has a facilitatory effect on LTH. Testing performance (6 trials). Ordinate: mean score per trial ± SEM. Injections after a weak training protocol (5 trials). *A*, Open bars: injection with vehicle; striped bars: idem with diverse doses of ANGIV. *B*, Idem but striped bars stand for ANGIV coinjected with different doses of saralasin (SARA). Intersession interval: 24 h. Other symbols as in Figure 4. *P<0.05 compared to control.

and drug (ACT or INH) groups was observed during training.

The above results demonstrated that the two cAMP analogues employed were able to alter LTH in the crab. However, in order to conclude that PKA is actually implicated in the formation of *Chasmagnathus* LTH, a biochemical characterization of the effects of both ACT and INH on the crab cAMP-dependent protein kinase was performed. *In vitro* kinase assays using extracts of *Chasmagnathus* brain showed a dose-dependent increase of phosphotransferase activity by ACT and an antagonist effect of INH. Addition of PKi, i.e., the specific inhibitor of PKA, clearly reduced the level of activity induced by ACT, thus indicating that the effect of the cAMP analogue was essentially due to PKA activation in the crab. To our knowledge, this was the first report in which the action of cAMP analogues has been studied by means of drug administration to intact animals.

Briefly, the results showed that PKA activation is a necessary step for LTH in *Chasmagnathus*, thus confirming that the cAMP mechanism of signal transduction seems to be a conserved molecular process implicated in long-lasting storage of memory.

Components of the renin-angiotensin system (RAS) are involved in LTH modulation

Manipulation of brain angiotensin levels appears to influence acquisition and recall of a newly learned task in vertebrates but results are still controversial (47-50). However, research on this subject has been confined to this phylum, despite the fact that components of RAS have been described in invertebrates: for example, angiotensin II and I in leeches (51,52), angiotensinogen-like epitopes in the nervous system of *Aplysia* (53) and angiotensin-converting enzyme-like activity in blue crabs (54), reflecting the high conservation of angiotensins in the course of evolution. Therefore, it seemed justified to explore the influence of angiotensins on *Chasmagnathus* LTH (32,35).

Human angiotensin II (ANGII) (50 pmol) injected immediately after a weak training protocol (10 trials) showed LTH at 24 h mainly when data analysis was confined to the initial testing phase. Such facilitatory effect of ANGII proved to be dose dependent and reversible by saralasin (5 pmol), i.e., by an ANGII antagonist with a well-known inhibitory effect on the whole current family of ANGII receptors (55). Moreover, the effect of ANGII vanished when the weak training protocol was reduced to only 5 trials or when the peptide was given before training or 1 h after. On the other hand, saralasin impaired LTH when injected before a strong training protocol, though no effect on STH was shown. An amnestic effect of saralasin was also obtained when the drug was given immediately after the strong training but not 1 h later.

Biochemical experiments provided additional support for the existence of RAS

components in *Chasmagnathus*. The brain showed ANGII-like immunoreactivity, estimated by RIA, and activity corresponding to angiotensin-converting enzyme, estimated by a significant hydrolytic activity of Hippuryl-His-Leu which was inhibited by 5 mM EDTA and 1 µM captopril (32). In addition, angiotensin-like immunoreactivity was detected by immunohistochemical techniques in cell bodies of neurons corresponding to the two main divisions of the *Chasmagnathus* brain, namely the supraesophageal ganglia and in the terminal medulla of the lateral optic lobes.

Recent results (56) showed that a weak training protocol of 5 trials managed to induce LTH when 5 pmol of the angiotensin II (3-8) fragment termed angiotensin IV (ANGIV) was injected post-training, suggesting that ANGIV has a facilitatory effect stronger than that obtained with ANGII. This effect proved to be dose dependent with a minimal effective dose near 0.5 pmol and reversible by saralasin (5 pmol) (Figure 6), a result which is in conflict with reports indicating that saralasin fails to reverse the effect of ANGIV (57). However, this disparity could be accounted for by assuming either that in this case a cleavage in the analogue molecule produces a fragment that competes with the AT4 receptor or that the amnestic effect of saralasin masks the facilitatory effect of ANGIV. Fifty pmol of the nonpeptide angiotensin receptor antagonists DUP and PD, selective for AT1 and AT2 receptor subtypes, respectively, failed to reverse the ANGIV effect when coinjected after weak training (5 trials) and, unlike saralasin, showed no amnestic effect when given after a strong training protocol (15 trials).

Two hypotheses can be offered (56) to account for the preceding results and to provide a theoretical framework for further research on the relation between ANGII and ANGIV with respect to crab memory. Both hypotheses assume that each peptide acts on different receptor subtypes.

According to a first view, ANGII or ANGIV may induce memory enhancement in crabs through different specific receptor subtypes. The action of ANGII would be mediated by receptors other than those described for mammals since neither DUP nor PD showed amnestic effects after a strong training protocol. It should be noted that no angiotensin receptors have been described as yet for invertebrates.

The second hypothesis proposes that the effects obtained with ANGII are a consequence of its cleavage to ANGIV, i.e., that ANGIV is the actual agent of the facilitatory effect of angiotensin on the long-term memory of *Chasmagnathus*. A basic assumption of this view is that the formation of crab ANGIV involves successive cleavages of the N-terminus from ANGII to ANGIV, as also observed in mammals (58).

STH may be mediated by endogenous opioids

An appreciable number of studies have been performed in our laboratory focusing on the probable role of opioids in STH (= drop in reactivity to the danger stimulus during the training session of a strong protocol), and in LTH (= retention of the habituated response after an intersession interval). Thus, it has been shown that morphine or the synthetic analog of Met-enkephalin, [D-Ala^2]methionine-enkephalin (DAME), produces a dose-dependent, naloxone-reversible reduction at the level of the escape response during training (33,34) and that this effect may be accounted for by a central action (59,60). In addition, two lines of evidence support the view that STH involves the modulatory action of an endogenous opioid mechanism. First, after naloxone pretreatment an enhancement in the escape response emerged over trials, slowing down the STH (13), and second, a strong training produced a transient, naloxone-reversible, inhibitory effect on the subsequent escape

response to a different type of stimulus presented immediately after training (61,62).

On the other hand, pre-training injection of opioids completely disrupted LTH (59) but post-training injection had no amnestic effect (60). Intriguingly, pre-training injection of GABA abolished the response during training but failed to show disruption of LTH, thus offering a new example of the so-called response-independent habituation (63). The disparity as regards LTH between results obtained with opioids (response abolition at training and disruption of LTH) and those obtained with GABA (response abolition at training and maintenance of LTH) is explained in the following terms. The depressing effect of exogenous opioids on the escape response to the phasic stimulus (= the danger stimulus) during training results from an impairment in perception, namely, from an interference with the decoding of the danger signal, while the similar effect of GABA comes from impairment of responsiveness, namely, from an inhibitory action on the efferent limb. Therefore, opioid-treated crabs fail to acquire LTH since they did not perceive the danger stimulus during training, while GABA-treated crabs acquire LTH since they perceive the danger stimulus though they are unable to run away.

No results are available concerning a possible role of opioids with respect to each of the putative memory components of LTH. However, it may be surmised that when the first testing trial recalls the signal memory, an endogenous opioid starts to accelerate the fall of reactivity in a similar way as an endogenous opioid would mediate the STH.

The dual-process hypothesis of LTH

Several intriguing and challenging questions arise from the results presented in this review concerning the dual-process hypothesis of LTH. Figure 7 summarizes the main results and questions. Behavioral and mechanistic properties attributed to initial memory are illustrated in the upper part of the figure and those attributed to the retraining memory are illustrated in the lower part. A question mark indicates results based on speculations. Ongoing experiments are dealing with two of these items. In one case the purpose is to ascertain if an endogenous opioid mediates the retraining memory and in the other the

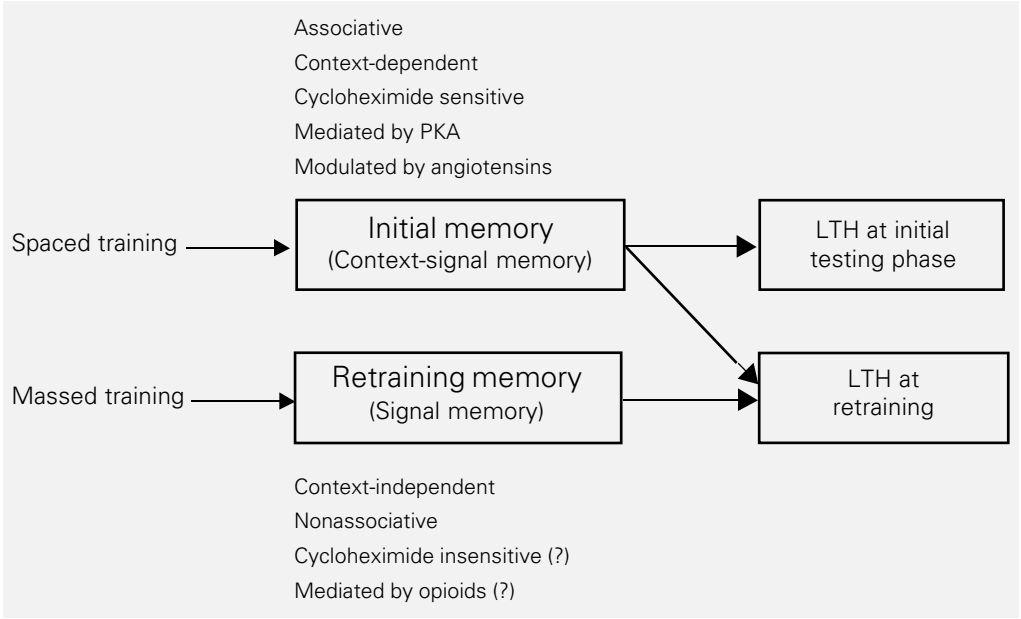

Figure 7 - The dual-process hypothesis of LTH. Schematic presentation of the main results and hypotheses. See text.

purpose is to explore how PKA activity, protein synthesis and angiotensin action are related to each other within a process subserving the initial memory.

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