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Report

The Octopus Vertical Lobe Modulates Short-Term Learning Rate and Uses LTP to Acquire Long-Term Memory

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Summary

Analyzing the processes and neuronal circuitry involved in complex behaviors in phylogenetically remote species can help us understand the evolution and function of these systems. Cephalopods, with their vertebrate-like behaviors [1–5] but much simpler brains [6], are ideal for such an analysis. The vertical lobe (VL) of *Octopus vulgaris* is a pivotal brain station in its learning and memory system [7]. To examine the organization of the learning and memory circuitry and to test whether the LTP that we discovered in the VL [8] is involved in behavioral learning, we tetanized the VL to induce a global synaptic enhancement of the VL pathway. The effects of tetanization on learning and memory of a passive avoidance task were compared to those of transecting the same pathway. Tetanization accelerated and transection slowed short-term learning to avoid attacking a negatively reinforced object. However, both treatments impaired long-term recall the next day. Our results suggest that the learning and memory system in the octopus, as in mammals [9], is separated into short- and long-term memory sites. In the octopus, the two memory sites are not independent; the VL, which mediates long-term memory acquisition through LTP, also modulates the circuitry controlling behavior and short-term learning.

Results

The octopus vertical lobe (VL) system lies dorsally in the central brain, allowing access for physiological and surgical manipulation (Figure 1A). In our slice preparation of the MSF-VL system, we discovered a robust long-term potentiation (LTP) of the glutamatergic synaptic inputs from the median superior frontal lobe (MSF) to the VL (Figure 1B, arrows) [3, 8]. Here, we present a method for inducing global LTP in the VL in vivo to examine the effect of artificial synaptic enhancement on learning and retention of a passive avoidance task. We compare the effects of global LTP with the effects of disconnecting the MSF input to the VL (Figures 1A and 1B, dashed line).

LTP was induced globally in the VL of anesthetized animals by high-frequency (HF) stimulation of the MSF-VL system (see the Supplemental Experimental Procedures available online). The effectiveness of this method was confirmed in the VL of brains isolated from three sham-operated and three tetanized animals (Figure 2). Three parameters were measured: (1) the synaptic field potential amplitude (fPSP, inset Figure 2B), (2) the residual LTP that could be induced (Figures 2A–2D), and (3) the dynamic of the fPSPs (Figures 2F and 2G).

The average fPSP amplitude in the tetanized brains was 2.05 times larger than in the sham-treated brains, but this difference was not significant in t test, probably due to the large variability in fPSPs amplitudes (0.103 ± 0.116 mV (SD), n = 21 versus 0.213 ± 0.258 mV, n = 20, p = 0.085). Significant difference is revealed in a nonparametric analysis of the fPSPs medians (p = 0.0256, Fisher's exact test of medians).

LTP was induced by four HF stimulation trains [8] at each of the sites marked by colored circles on the VL schema (Figures 2C and 2D), with the level of LTP being defined as the relative increase in a test fPSP amplitude (Figures 2A and 2B). To match the training protocol (Figure 1C), these measurements started ~1.5 hr after tetanization. LTP levels were quite variable in both groups (Figures 2C and 2D), but the level of LTP induced in the tetanized VLs was significantly lower than in the sham-treated brains (an average reduction of 56%, Figure 2E, p = 0.0458, t test). The tetanization did not occlude or completely saturate the LTP, and some locations in the tetanized VL maintained a robust LTP (Figure 2D, right).

We analyzed the dynamic properties of the fPSPs to ensure that the tetanization effect was not due to any tetanization-induced damage. Figure 2F shows examples of the first 7 of 20 fPSPs in the first of the four HF trains used to induce LTP ("4XHF," Figures 2A and 2B). The fPSPs demonstrated a more robust but slower synaptic facilitation during the train in the sham-operated example (blue trace) than in the tetanized brain (red trace). The dynamic differences between the tetanized and sham-operated brains become apparent when the amplitudes of the 20 fPSPs of the train are normalized at each recording site to the sum of these 20 fPSPs. This method most likely normalizes the variability in dynamic states of the release machinery [8].

Figure 2G gives the averages and standard error of the mean (SEM) of these normalizations. In the tetanized brains (red curve) the first fPSP was 66% larger than in the sham-operated brains (p = 0.0147, t test). (This means that more of the sum of the 20 fPSPs was "released" in the first fPSP.) In addition, the peak facilitation occurred earlier and the subsequent depression was faster in the tetanized brains. These differences in dynamics perfectly fit the typical changes in the fPSP dynamics after LTP induction [8] and therefore strongly suggest that the higher fPSPs amplitudes and lower level of LTP in the tetanized animals are indeed due to partial saturation of LTP.

For a behavioral test we chose the robust, simple, and fast training paradigm of a passive avoidance task, which is a form of learning that octopuses most likely use in nature (see Behavioral Experiments in Supp. Data and Figure 1C). Before the experiments the octopuses were pretrained to attack a white ball for a food reward. In the training session the octopuses were trained to avoid attacking a red ball by negative reinforcement with $12V_{ac}$ electric shocks (Movie S1). They were considered trained when they reached a criterion of

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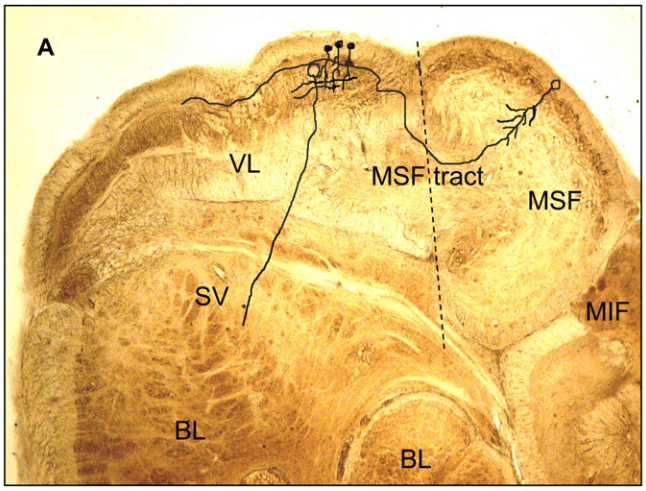

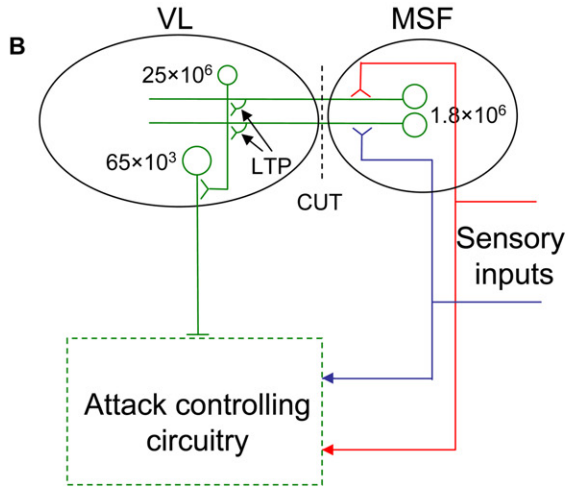

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Experimental procedures

Experimental group	N	acclimatization → >2 weeks	pre-training → 1-3 d before exp	anesthesia → 25-45 min	n	treatment → ~30 min	recovery → ~75 min	training → to criterion	testing → next day
control	5	√	√	-	5	-	-	√	√
MSF tract transection	21	√	√	√	9	sham cut	√	√	√
					12	cut	√	√	√
in-vivo tetanization	29	√	√	√	15	sham tetanized	√	√	√
					14	tetanized	√	√	√
non-contingent control	13	√	√	-	5	-	-	5 no-shock trials	-
					8	-	-	15 no-shock trials	-

Figure 1. The Organization of the MSF-VL System and the Experimental Procedures

(A) The unstained median sagittal section through the dorsal part of the supraesophageal brain mass with superimposed schematic drawing of the three types of neurons and their connections (see details in [B]). Abbreviations: MSF, median superior frontal lobe; VL, vertical lobe; MIF, median inferior frontal lobe (partial view); SV, subvertical lobe; BL, basal lobe. The MSF tract connects the two lobes.

(B) A schematic wiring diagram illustrates the connections, number of cell types [10], and possible inputs and outputs of the MSF-VL system. Arrows depict excitatory synaptic connections undergoing LTP. The dashed lines in (A) and (B) schematically marks where the MSF tract was transected.

(C) A flow diagram with details of the stages and time schedules of each experimental group (see details in the Supplemental Experimental Procedures). Abbreviations: N, number of animals in each group; n, the number of animals used for each experimental treatment.

four consecutive trials without touching the red ball. Readiness to touch the white ball was tested at the beginning and the end of the training session. A group of 13 pretrained animals (Figure 1C) was exposed to the red ball but without the shock and, thus, served as noncontingent controls.

The learning curves in Figure 3 give the percentage of animals touching the red ball as a function of the training trial number. Figure 3A shows that all the sham-operated animals generated similar learning curves, which did not differ from those of the five control animals (Figure 1C). Pretrained animals exposed to the red ball but without the shock (noncontingent controls) demonstrated a much slower rate of decline in touching the red ball (Figure 3A); habituation or fatigue appear to be slower than acquiring the passive avoidance task. Because the behavioral data are nonparametric, we used Fisher's exact test to check for differences between the cumulative number of touches versus no-touches from the second trial (see the "Statistical Procedures" section in the Supplemental Data). The differences between the various sham-operated and control groups were not significant, and the cumulative P values were greater than 0.1 (Figure 3A, bottom).

The 12 animals with transected MSF tracts needed significantly more training trials than did the nine sham-operated animals to reach criterion (10.17 ± 0.98 SEM) versus 4.89 ± 0.77, p = 0.0008, t test). The difference between the two curves (Figure 3B) is evident, and from the fifth trial the cumulative P is less than 0.01 (Figure 3B, bottom; Figure S3A; and see the Supplemental Experimental Procedures for an independent bootstrap simulation for this and the following experiments). Octopuses with transected MSF tracts mastered the task actively because they learned the task much faster than the reduction in number of ball touches showed by the noncontingent controls (Figure 3B). Thus, the input from the MSF to VL (which is most, if not all, of the MSF output [10]), is important, but not crucial, for short-term acquisition of the avoidance task. These results are similar to previous findings in which removal of VL or MSF or transecting the MSF tract impaired, but did not block, octopuses' associative learning not to attack a crab if the intertrial interval was short enough [11, 12]. All of these results support an inhibitory effect of the VL system on the visual/motor centers that drive the attack behavior.

Postmortem examinations showed that the amount of tract transected (between 50% to 90%, n = 10) was not significantly correlated with learning performance during training (Figure S5).

More than half of the animals with transected MSF tract attacked the positively rewarded white ball at the end of training

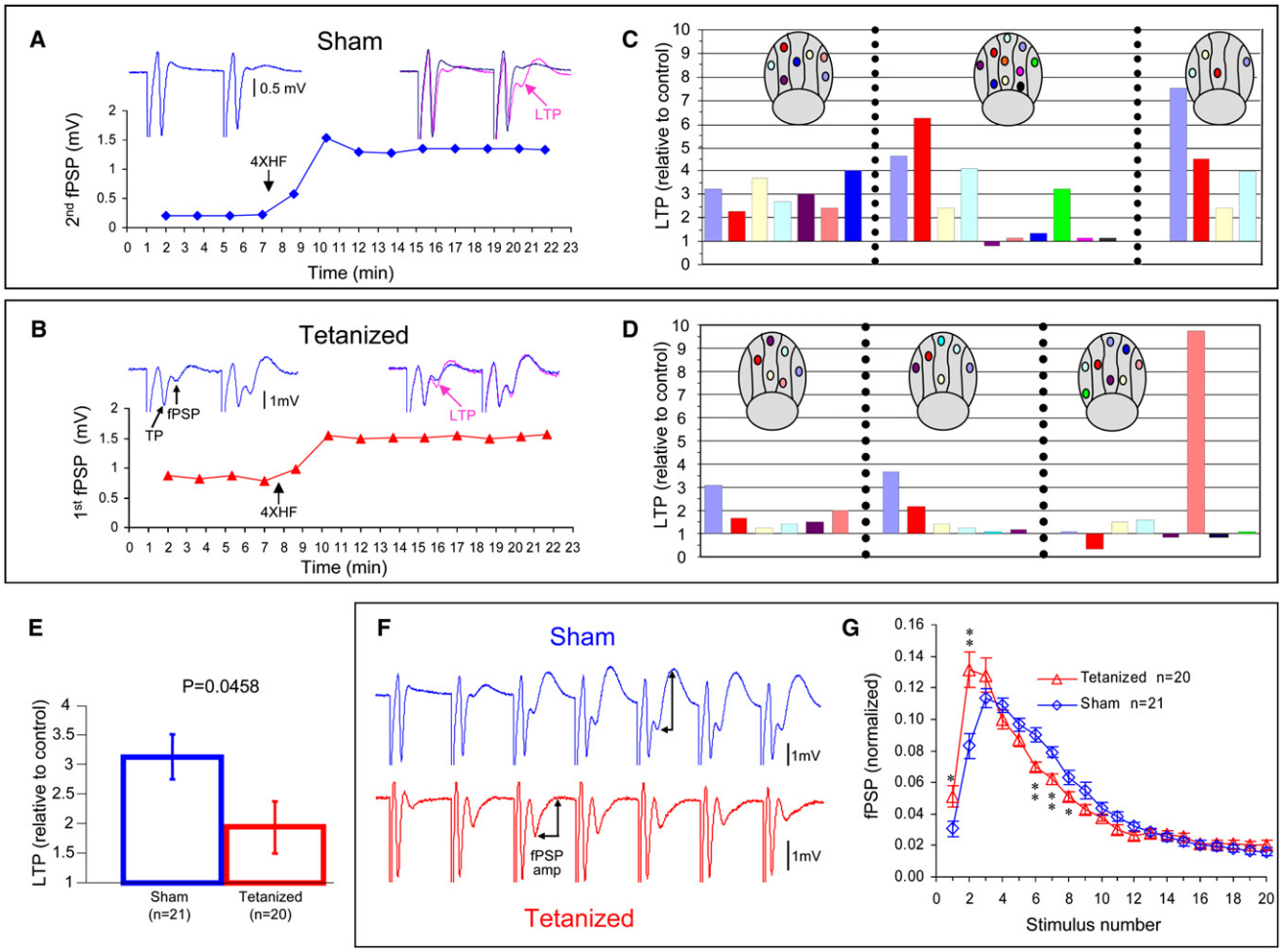

Figure 2. Testing the Effectiveness of the Tetanization in Brains Isolated from Tetanized and Sham-Treated Octopuses

(A) LTP is assessed in a sham-treated brain. The insets show records of field potentials evoked by twin pulses (20 ms interval, see inset in [B]) TP, tract potential. Because the first stimulus in this case caused no fPSP (left inset), the graph depicts the amplitude of the second fPSP (measured from averages of 10 responses). After four HF stimulation trains (4XHF), a marked facilitation is evident (see superimposed records in the right inset). The level of LTP is defined by the ratio between the fPSP amplitude at 10 min after HF to that in the control.

(B) Residual LTP is assessed in a tetanized brain. Details are similar to (A) except that the graph depicts the first fPSP (insets).

(C and D) Summary of the residual level of LTP in brains isolated from sham-operated (C) and tetanized (D) animals. The brains were glued within the experimental bath, and several sites, marked by different colors, were tested for the relative increase in synaptic field potential after HF stimulation, as exemplified in (A) and (B). The stimulating and recording electrodes were placed 1–1.5 mm apart along the longitudinal axis of the VL gyri. The levels of LTP are shown in the histograms in the respective color and in the order of testing from left to right.

(E) Tetanization reduced average LTP by 56% ($p = 0.0458$, t test). The error bars represent the SEM.

(F) Traces show examples of the facilitation and following depression of the fPSPs during the HF train (amplitude of the most facilitated fPSP is indicated by arrows). The first seven of the 20 (50 Hz) stimuli are shown.

(G) Averaged fPSPs are plotted in the first tetanization train at 21 recording sites in sham-operated brains (blue) and 20 in tetanized brains (red). The fPSPs at each recording site are normalized to the sum of amplitudes of the 20 fPSPs in the train. The differences between the two groups are typical of the changes induced by LTP in slice preparations (see text). *$p < 0.05$, **$p < 0.01$, t test. The error bars represent the SEM.

(8/12 operated and 5/9 sham-operated animals; $p = 0.6731$, Fisher's test), indicating that long-term memory of visual discrimination was not affected. (Only 2/8 transected and 0/5 sham-operated animals extended arms from their home to touch the white ball and thus might have sensed the bait). Thus, at least positively rewarded associations appear to be stored outside the VL system or their recall does not require MSF input to the VL. Muntz has suggested the optic lobes as a site for storing visual memory [13].

In contrast to transecting the MSF tract, tetanization did not slow down acquisition (Figure 3C). Surprisingly, the tetanized animals seemed to acquire the task faster than the sham-operated animals. Although there was no significant difference between the number of training trials to reach criterion (5.67 ± 0.99, n = 15 versus 4.21 ± 0.68, n = 14 sham-operated versus tetanized animals, respectively, $p = 0.2416$, t test), the first trial in which the tetanized animals did not touch the ball occurred significantly earlier than in the sham-treated animals (3.50 ± 0.23 trials versus 4.87 ± 0.60, $p = 0.0482$, t test). (There was no difference between when the transected and sham-treated animals stopped touching the ball.) The cumulative P of the running Fisher's test of the difference between the two acquisition curves reached less than 0.01 (0.0094) (Figure 3C, bottom, and Figure S3B). There was almost no difference until the third training trial, suggesting a similar initial tendency to attack the red ball.

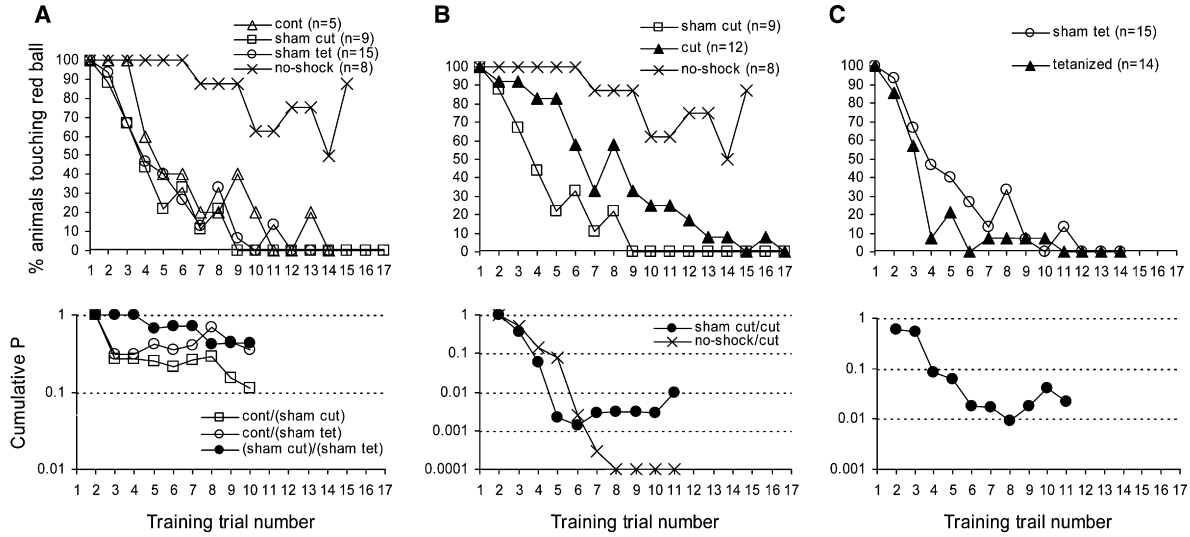

Figure 3. Transection Slows and Tetanization Enhances Short-Term Learning of the Avoidance Task

(A) Sham-operated and control (unoperated) animals show similar learning curves, and a cumulative Fisher's test revealed no significant differences between the groups (bottom; P in the n^{th} trial was calculated by Fisher's exact test of a 2 × 2 contingency table (two groups versus two outcomes), in which outcome 1 is the sum of touches and outcome 2 is the sum of no-touches made by the group from the 2^{nd} to the n^{th} trials. See the Supplemental Experimental Procedures). These curves differ from those from no-shock controls.

(B) The MSF-transected animals show significantly slower learning curves than the sham-controls, with a significant difference from the fourth testing trial onward (bottom). Nevertheless, the transected animals stopped touching the red ball significantly faster than no-shock controls (bottom).

(C) Tetanized animals learn faster than the sham-operated animals; by the eighth trial the level of cumulative Fisher's test fell below 0.01 (=0.0094) (bottom).

The short-term avoidance learning again appeared specific to the red ball, because at the end of training the tetanized animals tended to attack the white ball, behavior similar to that exhibited by sham control animals (11/15 sham-operated and 10/14 tetanized animals; only 2/11 sham-operated and 1/10 tetanized animals extended arms from their home to touch the baited ball). Older or positively reinforced memories thus do not appear to be stored in VL networks or, alternatively, global LTP does not erase already consolidated long-term memory. This finding also shows that unlike electroconvulsive shocks [14], tetanization does not generally suppress behavior.

The day after the training (Figure 1C) we checked for long-term memory of the task by presenting the red ball in five consecutive trials with a 5 min intertrial interval, as done in the training paradigm but without reinforcement shock. In contrast to the 13 noncontingent control octopuses, all touched the ball in the first trial (Figure 4, top panels), the one control and two

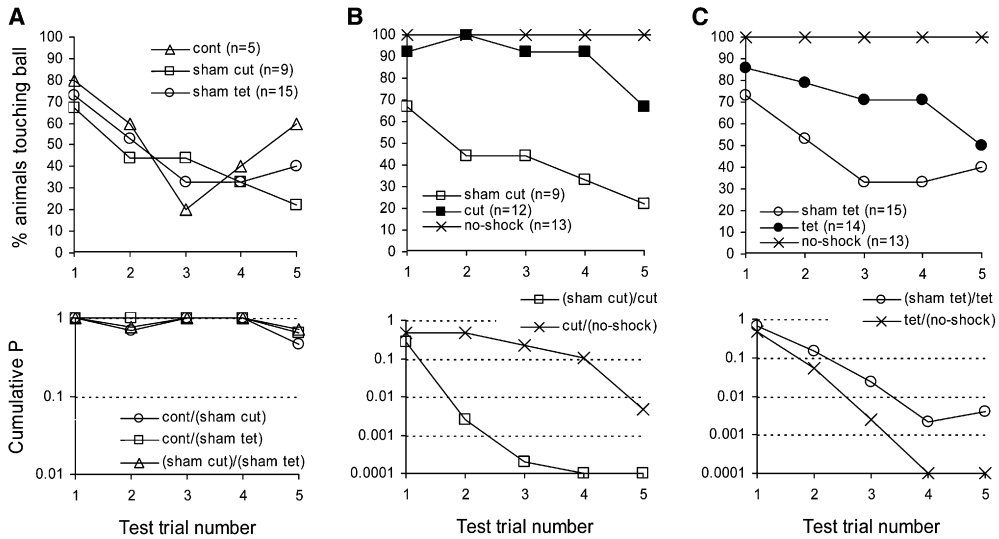

Figure 4. Tetanization and Transection Impair Long-Term Recall

As described in Figure 3 except that the animals were given five test trials without electric shock. Testing revealed no significant difference in long-term memory (i.e., first test trial, but see text) but impairment in recall in consecutive tests both in transected (B) and tetanized (C) animals. By the fifth test trial, the experimental animals showed some retention. Cumulative Fisher's exact test (see the legend for Figure 3) between the treated and the sham groups and the treated groups and the noncontingent controls are shown in the bottom panels.

sham-operated groups showed a significant difference from a hypothetical mean of 100% in a one-sample t test (73.33 ± 3.76%, t = 7.0989, df = 2, p = 0.0193). Therefore, there was some retention in the first test. The two experimental groups (transected = 92%, tetanized = 86%), on the other hand, were not statistically different from the 100% performance of the noncontingent controls, suggesting that both treatments impaired this long-term memory.

The following tests showed significant differences in the frequency of touching the ball between experimental and control animals (Figure 4). In contrast to the noncontingent controls that touched the red ball in each of the first five trials (n = 13, Figure 1C), the control group and both groups of sham-operated octopuses showed a reduction in the percentage of touching the ball in the second and third test trials (Figure 4A). This result demonstrates a behavior in which a conditioned stimulus without reinforcement can lead to either recall, fast reacquisition, or recovery from spontaneous extinction of a previously learned task. We cannot exclude, however, that the CS may contain some form of negative reinforcement because the unshocked red ball was pulled away when the animals attacked it (see Movie S1). This behavior resembles, to some extent, that described in other studies showing recovery of an actively extinguished conditioned response even without reinforcement (review [15]).

Very significantly, however, the octopuses with transected MSF tracts (Figure 4B, top) maintained a high level of mistakes until the fourth test and already by the second test the cumulative P was below 0.01 (Figure 4B, bottom, Figure S4A). This suggests that the input from the MSF is essential for long-term acquisition and/or recall. The finding that all 13 nonshocked animals continued to attack the red ball for at least five trials (Figure 4B, top) suggests that the reduction in response in the sham-operated and control animals was associated with a cognitive process and not simply with fatigue or habituation.

Similar to the MSF-transected animals and in contrast to their enhanced short-term learning, the tetanized animals lacked significant retention of the task in comparison to their sham-operated controls (Figure 4C, top, and Figure S4B). This lack of retention was less profound than in the MSF-transected animals; only by the fourth test did the cumulative P fall below 0.01 and it did not reach the low levels of the transected animals. In addition the cumulative difference from noncontingent control was highly significant in comparison to that of the transected animals (Figures 4B and 4C, bottom). The lower effect of tetanization than of transection may be explained by the incomplete saturation of LTP by the tetanization (Figure 2E).

It is unlikely that the impaired long-term memory in the tetanized animals is associated with receiving fewer shocks during their faster training (Figure 3C). First, there was no significant correlation between the number of training trials, either in sham-operated or tetanized animals, and the number of mistakes they made during the first three testing trials (Figure S6; we consider only the three first tests to avoid complications due to "erratic" behavior in the last tests). Second, most of the tetanized animals (10/14, versus 4/15 sham operated; p = 0.027, Fisher's test) made mistakes in each of the first three test trials (Figure S6).

As after short-term learning, long-term recall for the positively rewarded white ball was not affected by tetanization or transection. All 15 sham-operated versus 13/14 tetanized and 12 transected versus 7/9 sham-transected octopuses touched the white ball at the end of testing. (Only one animal touched the white ball by extending its arm.)

Discussion

Our study provides new insights into the organization of the learning and memory system of an invertebrate with advanced behavior. In simple forms of learning in the withdrawal reflexes of gastropod mollusks, both short- and long-term memory are localized in the same synaptic connections [16–18]. A more complex behavior in the mollusk Lymnaea indicates some separation between short- and long-term memory sites [19]. The octopus, like vertebrates [20], shows a clear separation between the sites of short- and long-term storage. Short-term memory apparently is consolidated within the "behavior controlling" circuitry [7, 21]), whereas long-term memory is retained (or depends) on a dedicated brain area—the VL system (Figure 1B). Insects, which also have complex learning abilities [22], also have evolved a special brain structure, the mushroom bodies, for these cognitive functions [23, 24]. Short- and long-term memory of nonreflexive behaviors thus appear to have a universal organization principle in which short-term traces are stored in the behavior-controlling circuitry separate from the site acquiring or controlling the consolidation of long-term memory traces.

Our results fit a simple feed-forward model for octopus avoidance-learning systems (see Figure 1B). The visual and aversive sensory inputs feed in parallel to the VL system and to the circuits controlling behavior. In the short term, the VL output inhibits behaviors associated with aversive experience (via the large efferent cells). In parallel, the VL uses activity-dependent LTP to acquire and consolidate these associations in long-term memory traces, conceivably in its matrix-like connections [7]. LTP in the VL apparently is not involved in the short-term association during the avoidance training because tetanization impairs only long-term retention. However, if indeed the VL output does inhibit the attack behavior [11, 12], tetanization may enhance this effect by amplifying synaptic connections in the VL [8] (Figure 2). This increase in inhibitory input to the circuits controlling behavior could accelerate short-term avoidance learning similar to the action of an inhibitory neuromodulator on a cellular correlate of habituation in the mollusk *Aplysia* [25].

From an evolutionary standpoint, our results join other studies supporting LTP as a universal process for the mediation of long-term behavioral memory. We still do not know if similar molecular and cellular mechanisms drive the activity-dependent synaptic enhancement. Although VL LTP has several Hebbian characteristics, it is mediated by presynaptic modifications and is most likely independent of NMDA-receptors [8]. Interestingly, in gastropod mollusks [26, 27] and insects [28], NMDA-dependent plasticity is conserved [29]. It is possible that as in the hippocampus, which shows both NMDA-dependent and -independent plasticity, other synaptic connections in the VL or different areas in the octopus brain may use NMDA-dependent mechanisms. This is not unlikely because NMDA-like receptors have been identified immunohistochemically in cephalopod brains [30] and physiologically in chromatophore muscle cells [31].

Our octopus results fit findings in mammals that physiological saturation of LTP impairs long-term memory without impairing short-term learning [32]. Such experiments were proposed as one of the criteria for confirming the involvement of LTP in long-term memory [33]. In the saturation experiments in mammals, only complete saturation affects learning, but in the octopus even partial saturation impairs long-term memory. Few studies in mammals have demonstrated that LTP

saturation accelerates, rather than inhibits, associative learning [34] as found in the current study for short-term learning. Our results suggest that such contrasting outcomes may depend on the site of tetanization relative to the organization of the learning and memory system.

Our analysis of learning and memory in the octopus also supports the universality of a "modal" model of learning and memory in which short- and long-term memory are two separate processes, as suggested by Atkinson and Shiffrin [35] and Hebb [36]. However, the results in the octopus suggest a novel mode of interaction between long- and short-term memory systems, whereby the output of the long-term memory site (VL) modulates the circuits controlling behavior and short-term memory.

This organization of two separate memory systems and the supervisory effects of the VL on the behavior per se suggest that the long-term memory acquisition site has an additional capacity of evaluating "risks."

Supplemental Data

Supplemental Experimental Procudures, six figures, and one movie are available at http://www.current-biology.com/cgi/content/full/18/5/337/DC1/.

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