Memory reconsolidation and its maintenance depend on L-voltage-dependent calcium channels and CaMKII functions regulating protein turnover in the hippocampus

Weber Cláudio Da Silva[a,b,c,d,1], **Gabriela Cardoso**[a], **Juliana Sartori Bonini**[a,d], **Fernando Benetti**[a,b], and **Ivan Izquierdo**[a,b,1]

[a]Centro de Memória, Instituto do Cérebro, Pontifícia Universidade Católica do Rio Grande do Sul, RS90610-000, Porto Alegre, Brazil; [b]Instituto Nacional de Neurociência Translacional, Conselho Nacional de Desenvolvimento Científico e Tecnológico, RS90610-000, Porto Alegre, Brazil; [c]Programa de Pós-Graduação em Ciências Médicas, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, RS90035-003, Porto Alegre, Brazil; and [d]Laboratório de Neuropsicofarmacologia, Departamento de Farmácia, Universidade Estadual do Centro-Oeste, PR85040-080, Guarapuava, Brazil

Immediate postretrieval bilateral blockade of long-acting voltage–dependent calcium channels (L-VDCCs), but not of glutamatergic NMDA receptors, in the dorsal CA1 region of the hippocampus hinders retention of long-term spatial memory in the Morris water maze. Immediate postretrieval bilateral inhibition of calcium/calmodulin-dependent protein kinase (CaMK) II in dorsal CA1 does not affect retention of this task 24 h later but does hinder it 5 d later. These two distinct amnesic effects are abolished if protein degradation by proteasomes is inhibited concomitantly. These results indicate that spatial memory reconsolidation depends on the functionality of L-VDCC in dorsal CA1, that maintenance of subsequent reconsolidated memory trace depends on CaMKII, and these results also suggest that the role played by both L-VDCC and CaMKII is to promote the retrieval-dependent, synaptically localized enhancement of protein synthesis necessary to counteract a retrieval-dependent, synaptic-localized enhancement of protein degradation, which has been described as underlying the characteristic labilization of the memory trace triggered by retrieval. Thus, conceivably, L-VDCC and CaMKII would enhance activity-dependent localized protein renewal, which may account for the improvement of the long-term efficiency of the synapses responsible for the maintenance of reactivated long-term spatial memory.

Memory maintenance or persistence depends on the degree of emotional arousal present at the time of consolidation (1, 2), and apparently on several different biochemical and behavioral variables over the next several hours or days (3–9), especially in the hippocampus, which is widely recognized as the region in charge of consolidation and maintenance (10–13). An important factor in memory maintenance is reconsolidation (14–16), which originates in the labilization of memories caused by nonreinforced retrieval. Reconsolidation is a protein synthesis-dependent mechanism without which traces become progressively weaker (5, 14–19). In most cases, it occurs more readily the first few times that memories are retrieved (3). Otherwise, retrieval triggers extinction, which requires NMDA glutamate receptors and protein synthesis in the hippocampus, the basolateral amygdala, and the ventromedial prefrontal cortex (20–22). The relative predominance of extinction over reconsolidation or vice versa depends on so-called "boundary" conditions, which, aside from the recency of previous training mentioned above, are poorly understood (10, 14, 23–26). Kaang and his coworkers (27–29) have produced evidence of increased proteasome-mediated postsynaptic protein destruction in hippocampus upon retrieval, which would underlie the labilization of the trace at that time (27). Indeed, available evidence suggests that a crucial boundary condition in the extinction/reconsolidation dichotomy may be a balance between synaptic protein degradation and synthesis. The direction of this balance could be regulated by the extracellular-regulated kinase pathway, which, once activated by retrieval (30), participates in extinction but not in reconsolidation (31).

Several of the mechanisms underlying consolidation, reconsolidation, and maintenance depend on intracellular calcium level,

$[Ca^{2+}]_i$: for example, the activity of two major protein kinase families, the protein kinase C family (32) and the calcium/cal-modulin-dependent kinases (CaMKs). The most important of the latter is α-CaMKII (33–36). An important source of $[Ca^{2+}]_i$ are the long-acting voltage-dependent calcium channels (L-VDCCs), which have been proposed to play some role in memory processes (37–40). CaMKII has many purported or demonstrated roles in consolidation and perhaps in maintenance (10); one that has been recently suggested is to serve as a scaffold for proteasomes to act on dendritic spines (41). Several data also suggest a key role for proteasome activity in posttraining memory processing (27–29) up to at least 7 h after consolidation (42).

Here, we study pharmacologically the role of L-VDCCs, proteasomes, and CaMKII on the making, reconsolidation, and maintenance of spatial memories in a Morris water maze. This task has long been known to require the hippocampus (5, 43, 44), particularly through the induction and development of long-term potentiation (LTP) in that structure. Recent studies have extended the requirement of hippocampal LTP to the consolidation of a variety of other learning tasks, spatial and nonspatial (10–12, 22, 45, 46), which widens the scope of studies like the present one for the understanding of memory processes. The mechanisms of hippocampal LTP and memory formation are identical or very nearly so for several memory types (10, 12).

Results

Effect of NMDA Receptor Blockade on Reconsolidation of Long-Term Spatial Memory. As a major general control experiment, first we determined whether NMDA receptors are required in the hippocampus for reconsolidation of spatial memory in the Morris water maze task (MWM) using a spaced and strong training protocol (47). Rats trained for 5 d were submitted to a probe test in the absence of the escape platform 24 h after the last training session. Immediately after the probe test, the animals received bilateral infusions of vehicle or D(−)-2-amino-5-phosphono-pentanoic acid (AP5) (25 nmol per side) in the dorsal hippocampus. Retention was evaluated in a second probe test carried out 24 h or 5 d after the first one. AP5 had no effect on retention when infused into CA1 immediately after the first probe test, regardless of the time elapsed between the two tests (Fig. S1 *A–D*).

Effect of Blockade of L-VDCCs on Reconsolidation of Long-Term Spatial Memory. To investigate whether inhibition of L-VDCCs after spatial memory retrieval affects reconsolidation of the

reactivated trace, rats trained for 5 d in the spatial version of the MWM as above were submitted to a probe test in the absence of the escape platform 24 h after the last training session. Immediately after the probe test, the animals received bilateral infusions of vehicle or of the L-VDCC blocker nifedipine (NIFE) (10 nmol per side) in the dorsal hippocampus. Retention was evaluated in a second probe test carried out 24 h or 5 d after the first one. When given immediately after the first probe test (P1), NIFE significantly increased the latency to swim over the previous location of the escape platform [$t_{(19)} = 2.132, P < 0.05$ for P2 24 h after P1 (Fig. 1A); and $t_{(19)} = 3.663, P < 0.01$ for the second probe test (P2) 5 d after P1 (Fig. 1B)] and reduced to chance level the time spent in the target quadrant during the second probe test, regardless of the time elapsed between the two tests [$t_{(19)} = 3.625, P < 0.01$ for P2 24 h after P1 (Fig. 1C); and $t_{(19)} = 2.424, P < 0.05$ for P2 5 d after P1 (Fig. 1D)]. Additionally, NIFE did not affect spatial memory when given into dorsal CA1 24 h after the last training session in the absence of a behaviorally relevant event (Fig. S2 A and B) or when administered immediately after a test session in the presence of the escape platform 24 h after training (Fig. S2 C and D).

The amnesic effect of NIFE was entirely reversible. In fact, animals that had received intrahippocampal NIFE immediately
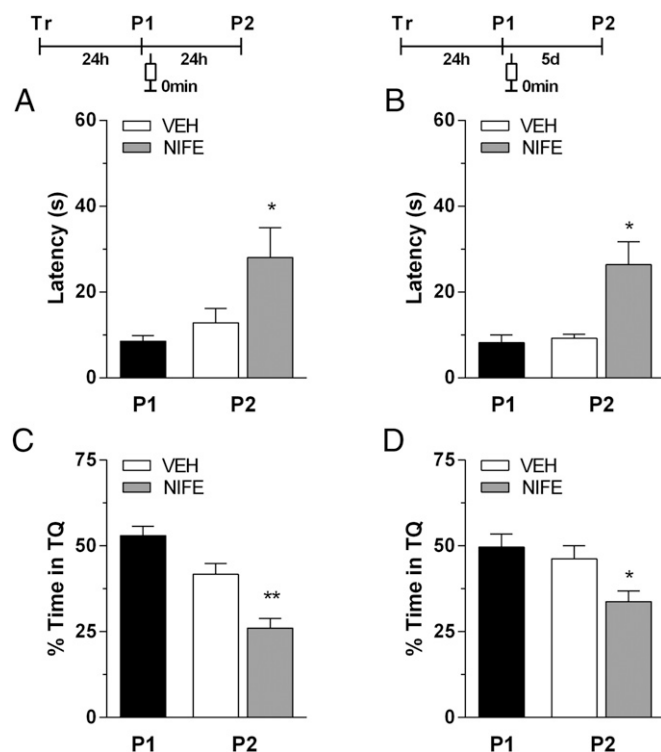

Fig. 1. Intrahippocampal infusion of NIFE immediately after nonreinforced retrieval hinders spatial memory retention as measured 24 h or 5 d after reactivation. Animals with infusion cannulae implanted in the CA1 region of the dorsal hippocampus were trained during 5 d in the spatial version of the MWM. Twenty-four hours after the last training session, the animals were randomly assigned to one of four experimental groups and submitted to a 60-s probe test in the absence of the escape platform (P1) (black bar). Immediately after P1, the animals received intrahippocampal infusions of vehicle (VEH) (white bar) or NIFE (10 nmol per side; gray bar). Memory retention was assessed in a second 60-s probe test (P2) carried out 24 h (A and C) or 5 d after P1 (B and D). Data are expressed as means (± SEM) of the latency to swim over the previous location of the escape platform (A and B) or as the percentage of swimming time spent in the target quadrant (TQ) (C and D). *$P < 0.05$ and **$P < 0.01$ vs. VEH in Student t test ($n = 9$–12 per group).

after P1 acquired the spatial preference to another platform location as consistently as did control animals (Fig. S3).

Effect of Inhibition of CaMKII on Reconsolidation of Long-Term Spatial Memory. To investigate whether inhibition of CaMKII after spatial memory retrieval affects reconsolidation of the reactivated trace, rats trained for 5 d in the spatial version of the MWM as above were submitted to a probe test in the absence of the escape platform 24 h after the last training session. Immediately, 30 min, or 90 min after the probe test, the animals received bilateral infusions of vehicle or autocamtide-2–related inhibitory peptide (AIP) (1.0 nmol per side) in the dorsal hippocampus. Retention was evaluated in a second probe test carried out 24 h or 5 d after the first one. When given immediately after the first probe test, AIP had no effect on retention if the second probe test was 24 h after the first one (Fig. 2 A and B). However, if the second probe test was 5 d after the first one, AIP caused a significantly increased the latency to swim over the previous location of the escape platform [$t_{(16)} = 2.262, P < 0.05$ for P2 5 d after P1; Fig. 2C] and reduced to chance level the time spent in the target quadrant during the second probe test [$t_{(16)} = 2.627, P < 0.05$ for P2 5 d after P1; Fig. 2D]. This result indicates that inhibiting CaMKII immediately after the reactivation of a long-term spatial memory did not hinder the subsequent reconsolidation but, instead, prejudiced the maintenance of the reconsolidated memory, so that this memory trace was no longer retrieved 5 d after the reactivation.

When AIP was given immediately after the first probe test carried out 5 d after the last training session, again, there was no amnesic effect if the second probe test was 24 h after the first one (Fig. 3 A and B), but there was an amnesic effect if a second probe test carried out 5 d after the first one. In this second probe test, AIP significantly increased the latency to swim over the previous location of the escape platform [$t_{(19)} = 2.178, P < 0.05$ for P2 5 d after P1; Fig. 3C] and reduced to chance level the time spent in the target quadrant during the second probe test [$t_{(19)} = 3.880, P < 0.01$ for P2 5 d after P1, Fig. 3D]. Thus, this results show that the prejudicial effect of CaMKII inhibition, immediately after the reactivation of a long-term spatial memory, on the maintenance of reconsolidation of this trace memory is independent of how long is the time elapsed between the first probe test and the last training session.

There was no effect of AIP, either on reconsolidation or on maintenance of reconsolidated long-term spatial memory, if AIP was bilaterally infused into the dorsal hippocampus 30 min (Fig. S4 A–D) or 90 min after the first probe test (Fig. S4 E–H). Therefore, the effect of AIP on maintenance of reconsolidated long-term spatial memory is restricted to a narrow time window soon after the reactivation of this memory trace.

Finally, AIP did not affect spatial memory when given into dorsal CA1 24 h after the last training session in the absence of a behaviorally relevant event (Fig. S2 A and B) or when administered immediately after a test session in the presence of the escape platform carried out 24 h after training (Fig. S2 C and D).

The amnesic effect of AIP was entirely reversible. In fact, animals that had received intrahippocampal AIP immediately after P1 acquired the spatial preference to another platform location as consistently as did control animals (Fig. S3).

Role of Protein Turnover on the Reconsolidation and Maintenance of Reconsolidated Long-Term Spatial Memory-Respective Dependence on L-VDCC and CaMKII Activities and Protein Synthesis. To investigate the role of protein degradation on the effect of blockade of L-VDCC on the long-term spatial memory reconsolidation, on the effect of inhibition of CaMKII activity on the maintenance of the reconsolidated long-term spatial memory, and also on the effect of inhibition of protein synthesis on the long-term spatial memory reconsolidation, rats trained for 5 d in the spatial version of the MWM as above were submitted to a probe test in the
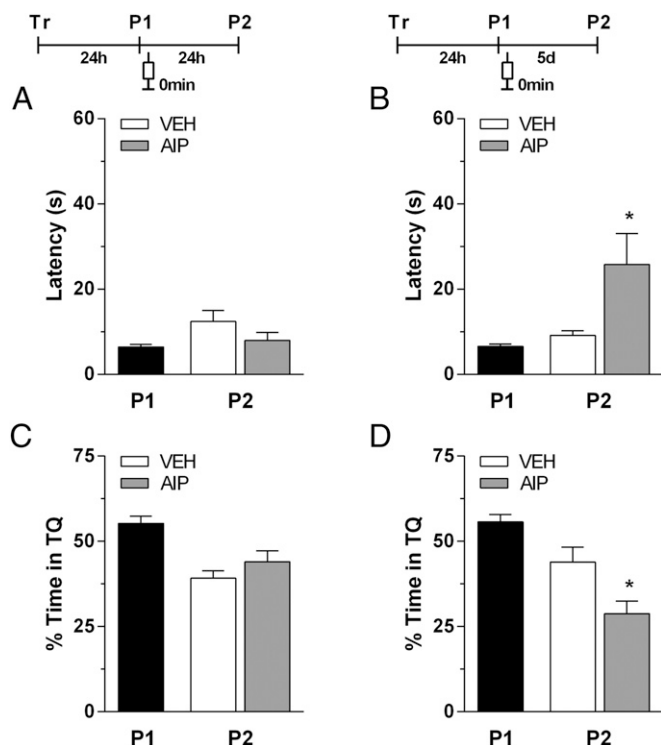
Fig. 2. Intrahippocampal infusion of AIP immediately after nonreinforced retrieval hinders spatial memory retention only if measured 5 d after reactivation. Animals with infusion cannulae implanted in the CA1 region of the dorsal hippocampus were trained during 5 d in the spatial version of the MWM. Twenty-four hours after the last training session, the animals were randomly assigned to one out of four experimental groups and submitted to a 60-s probe test in the absence of the escape platform (P1) (black bar). Immediately after P1, the animals received intrahippocampal infusions of vehicle (VEH) (white bar) or AIP (1.0 nmol per side; gray bar). Memory retention was assessed in a second 60-s probe test (P2) carried out 24 h (*A* and *C*) or 5 d after P1 (*B* and *D*). Data are expressed as means (± SEM) of the latency to swim over the previous location of the escape platform (*A* and *B*) or as the percentage of swimming time spent in the target quadrant (TQ) (*C* and *D*). *P < 0.05 vs. VEH in Student *t* test (n = 9 per group).

absence of the escape platform 24 h after the last training session. Immediately after the probe test, the animals received bilateral infusions of vehicle or anisomycin (ANI) (1.0 μmol per side), clasto-lactacystin β-lactone (βLAC) (200 pmol per side), βLAC plus ANI (200 pmol plus 1.0 μmol per side), βLAC plus NIFE (200 pmol plus 10 nmol per side), or βLAC plus AIP (200 pmol plus 1.0 nmol per side) in the dorsal hippocampus. Retention was evaluated in a second probe test carried out 24 h or 5 d after the first one. When given immediately after the first probe test, ANI significantly increased the latency to swim over the previous location of the escape platform [$F_{(3,28)} = 4.305$, $P < 0.05$ for P2 24 h after P1, ANI vs. vehicle (Fig. 4*A*); and $F_{(3,29)} = 7.589$, $P < 0.001$ for P2 5 d after P1, ANI vs. vehicle (Fig. 4*B*)] and reduced to chance level the time spent in the target quadrant during the second probe test, regardless of the time elapsed between the two tests [$F_{(3,28)} = 4.335$, $P < 0.05$ for P2 24 h after P1, ANI vs. vehicle (Fig. 4*C*); and $F_{(3,29)} = 5.455$, $P < 0.05$ for P2 5 d after P1, ANI vs. vehicle (Fig. 4*D*)]. βLAC, βLAC plus ANI, βLAC plus NIFE, or βLAC plus AIP had no effect on retention when infused into CA1 immediately after the first probe test, regardless of the time elapsed between the two tests (Fig. 4 *A–D*). Thus, the amnesic effects on reconsolidation caused by blockade of L-VDCC or inhibition of protein synthesis, and on maintenance of reconsolidated long-term spatial memory caused by inhibition of CaMKII, are cancelled by inhibition of proteasome activity. Furthermore,

the inhibition of proteasome activity per se is not necessary either for reconsolidation or for maintenance of reconsolidated long-term spatial memory.

The amnesic effect of ANI was entirely reversible. In fact, animals that had received intrahippocampal ANI immediately after P1 acquired the spatial preference to another platform location as consistently as did control animals (Fig. S3).

Discussion

Memory labilization at the time of retrieval has been explained by proteasome-mediated protein degradation (27), but it also coexists with at least two major protein synthesis-dependent processes: extinction (20, 22) and reconsolidation (14, 15, 23, 48). It is reasonable to think that retrieval triggers or is based upon a reorganization of the synaptic protein structure, where both protein destruction (in the labilization) and construction (in the subsequent reconsolidation) play a role (28, 49). CaMKII, like other signaling pathways [protein kinase C (32), the protein kinase C variant PKMZ (50), and cAMP-dependent protein kinase (13, 51–53)], might be important to signalize the protein synthesis increase and to redirect the newly synthetized proteins to the more active synapses (54–56). This multiple regulation might ensure the use of new proteins for retrieval in the presence of eventually reduced activity of one of the signaling pathways (13,
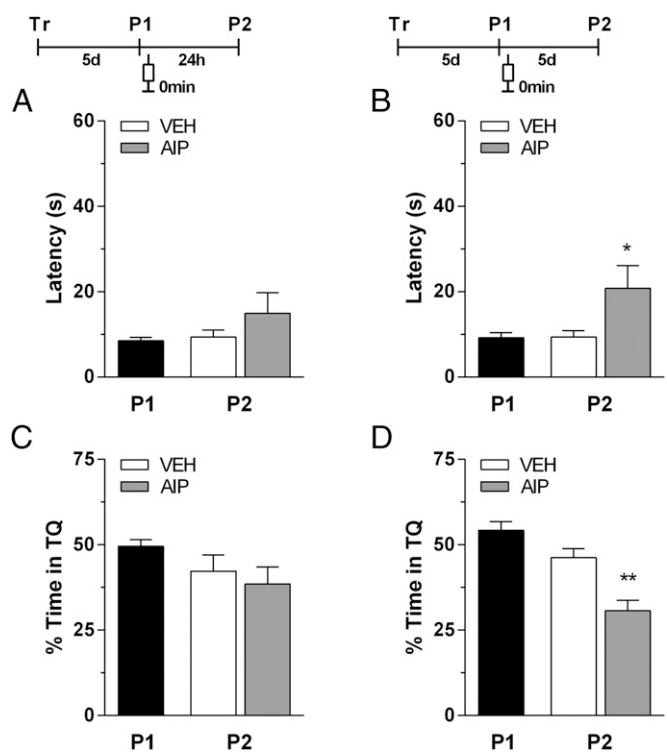

Fig. 3. Intrahippocampal infusion of AIP immediately after nonreinforced remote retrieval hinders spatial memory retention only if measured 5 d after reactivation. Animals with infusion cannulae implanted in the CA1 region of the dorsal hippocampus were trained during 5 d in the spatial version of the MWM. Five days after the last training session, the animals were randomly assigned to one of four experimental groups and submitted to a 60-s probe test in the absence of the escape platform (P1) (black bar). Immediately after P1, the animals received intrahippocampal infusions of vehicle (VEH) (white bar) or AIP (1.0 nmol per side; gray bar). Memory retention was assessed in a second 60-s probe test (P2) carried out 24 h (*A* and *C*) or 5 d after P1 (*B* and *D*). Data are expressed as means (± SEM) of the latency to swim over the previous location of the escape platform (*A* and *B*) or as the percentage of swimming time spent in the target quadrant (TQ) (*C* and *D*). *P < 0.05 and **P < 0.01 vs. vehicle in Student *t* test (n = 10–12 per group).
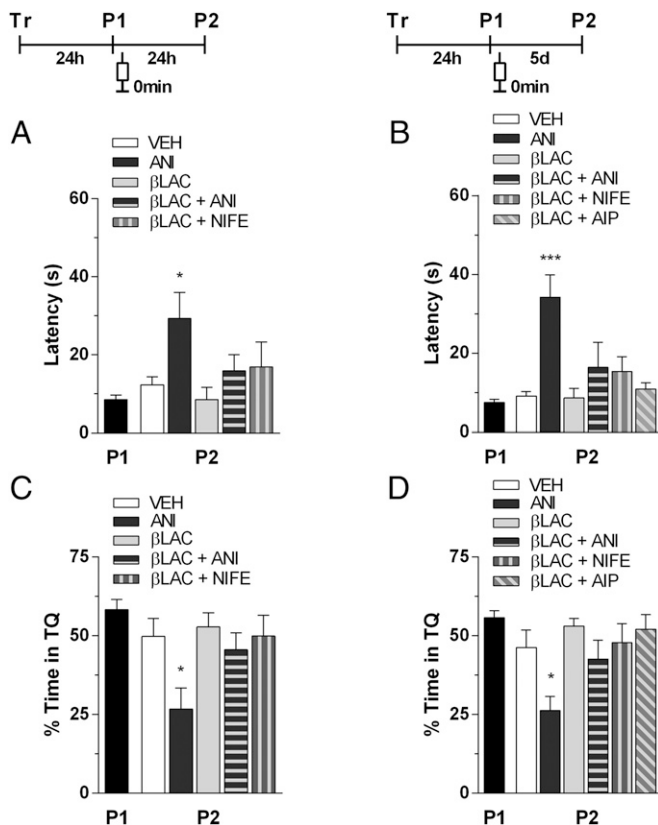
Fig. 4. The amnesic effect induced by the intrahippocampal infusion of ANI, NIFE, or AIP is blocked by proteasome antagonist. Animals with infusion cannulae implanted in the CA1 region of the dorsal hippocampus were trained during 5 d in the spatial version of the MWM. Twenty-four hours after the last training session, the animals were randomly assigned to one out of eleven experimental groups and submitted to a 60-s probe test in the absence of the escape platform (P1) (black bar). Immediately after P1, the animals received intrahippocampal infusions of vehicle (VEH) (white bar), ANI (1.0 µmol per side; dark gray bar), clasto-lactacystin βLAC (200 pmol per side; light gray bar), βLAC plus ANI (200 pmol and 1.0 µmol per side, respectively; horizontally striped bar), βLAC plus NIFE (200 pmol and 10 nmol per side, respectively; vertically striped bar), or βLAC plus AIP (200 pmol and 1.0 nmol per side, respectively; diagonally striped bar). Memory retention was assessed in a second 60-s probe test (P2) carried out 24 h (*A* and *C*) or 5 d after P1 (*B* and *D*). Data are expressed as means (± SEM) of the latency to swim over the previous location of the escape platform (*A* and *B*) or as the percentage of swimming time spent in the target quadrant (TQ) (*C* and *D*). *$P < 0.05$ and ***$P < 0.001$ vs. vehicle in Dunnett's test after one-way ANOVA ($n = 8$–9 per group).

53). Certainly, several authors (27–29, 49, 57) have proposed that the most likely variable underlying the process of reconsolidation-mediated maintenance is a balance between protein degradation and protein synthesis, rather than any of the two alone. Artinian et al. (48) showed that the inhibition of proteasomes leads to anterograde amnesia; in the present study, it did not. This might be explained by the different task/species combination used by both groups.

The importance of reconsolidation for persistence, and not (necessarily) for further retrieval shortly afterward, is stressed by the facts that several of the drugs studied by postretrieval administration [NIFE (Fig. 1) and AIP (Fig. 2)] showed an effect when the animals were tested 5 d but not just 24 h later. It is likely that mechanisms other than reconsolidation secure retrieval shortly after reconsolidation (3, 10). The present findings attest to the long-lasting nature of reconsolidation effects (17).

Here, we show the link between membrane events and intracellular processes that regulate protein turnover in the maintenance of reconsolidated memory. We demonstrate that this maintenance depends on specific biochemical mechanisms. It is hoped that future research will help to understand this relation. In particular, the factor(s) that trigger proteasomal protein degradation must be better defined.

The present findings might be taken to agree with the recent proposition by Ramachandran and Frey (58) that CaMKII activation, through an influence on actin dynamics during a synaptic capture process such as would accompany a reexposure to water maze learning (22, 46, 55), might play a role in LTP maintenance relevant to spatial memory maintenance. This type of memory is, in fact, the one most traditionally linked to LTP (5, 43).

Recent evidence points to the involvement of different CaMKs in memory processes (55). These enzymes are believed to act by clustering mechanisms that have recently been described (54). The differential timing of these mechanisms and of their linkage to synaptic tagging and capture processes (55) might explain differences in the time course of some of their effects, such as the one on consolidation and early memory maintenance, which is immediate (13, 33), and the one described here on reconsolidation, which is delayed (Fig. 3). The balance between protein synthesis and destruction (42) is activity-dependent (57) and, as discussed above, relies on CaMKII, which redirects proteins to the synapses more active at the time (55). Certainly, the activity that follows after consolidation and after retrieval is different (10, 24). Retrieval induces reconsolidation better 1 or 2 d after consolidation than later (3). Further experiments might elucidate this question.

Materials and Methods

Training in the Spatial Version of the MWM Learning Task. The water maze was a black circular pool (200 cm in diameter) conceptually divided into four equal imaginary quadrants for the purpose of data analysis. The water temperature was 21–23 °C. Two centimeters beneath the surface of the water and hidden from the rat's view was a black circular platform (12 cm in diameter). It had a rough surface, which allowed the rats to climb onto it easily once detected.

The swimming path of the animals was recorded using a video camera mounted above the center of the pool and analyzed using a video tracking and analysis system. The water maze was located in a well-lit white room with several posters and other distal visual stimuli hanging on the walls to provide spatial cues. Rats were handled 5 min per day for 3 d before training. Training using the spaced training protocol was carried out during 5 successive days (47). On each day, rats received eight consecutive training trials, during which the hidden platform was kept in a constant location. A different starting location was used on each trial, which consisted of a swim followed by a 30-s platform sit. Any rat that did not find the platform within 60 s was guided to it by the experimenter. Memory retention was evaluated in a 60-s probe trial carried out in the absence of the escape platform 24 h or 5 d after the last training session. To evaluate the effect of drugs given after memory reactivation, rats were trained for 5 d as indicated earlier, before being submitted to the first probe test in the absence of the escape platform 24 h or 5 d after the last training session. At different times after that, rats received intra-CA1 infusions of the drug under scrutiny or vehicle. Memory retention was evaluated in a second probe test carried out at 24 or 5 d after the first one.

Statistical Analysis. Data were analyzed by a two-tailed Student t test or ANOVA followed by Dunnett's post hoc tests, as appropriate.

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