Synaptic Protein Degradation Underlies Destabilization of Retrieved Fear Memory
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(*14*): a helical-shaped LEV starting at the inner wing, increasing in size along the wingspan, and finally connecting to the tip vortex (*9*, *27*); a cylindrical-shaped LEV that expands across the thorax and is connected to the two tip vortices (*6*, *14*); and a LEV that is connected to a small root vortex and a large tip vortex (*5*). The vortex system proposed here (Fig. 4) is most similar to the latter case.

The sharp leading edge of the bat wing probably facilitates the generation of the LEV (*21*), whereas the ability to actively change the wing shape and camber (*32*) could contribute to the control and stability of the LEV.

LEVs have now been observed in active unrestricted bat flight, with a strength that is important to the overall aerodynamics. Unsteady aerodynamic mechanisms for enhanced lift are therefore not unique to insect flight, and larger animals adapted for slow and hovering flight, such as these nectar-feeding bats, can (and perhaps must) use LEVs to enhance flight performance.

References and Notes

1. F.-O. Lehmann, *Naturwissenschaften* **91**, 101 (2004).
2. Quasi–steady-state wing theory assumes that the forces on a moving wing are equivalent to the sum of the forces on a fixed wing over a sequence of attitudes that track the wing motion. This model neglects acceleration forces and unsteady aerodynamic effects.
3. M. H. Dickinson, F.-O. Lehmann, S. P. Sane, *Science* **284**, 1954 (1999).
4. T. Weis-Fogh, *J. Exp. Biol.* **59**, 169 (1973).
5. T. Maxworthy, *J. Fluid Mech.* **93**, 47 (1979).
6. R. B. Srygley, A. L. R. Thomas, *Nature* **420**, 660 (2002).
7. C. Ellington, *Philos. Trans. R. Soc. London Ser. B* **305**, 1 (1984).
8. S. Vogel, *Life in Moving Fluids* (Princeton Univ. Press, Princeton, NJ, 1994).
9. C. P. Ellington, C. van den Berg, A. P. Willmott, A. L. R. Thomas, *Nature* **384**, 626 (1996).
10. T. Maxworthy, *J. Fluid Mech.* **587**, 471 (2007).
11. M. W. Luttges, in *Frontiers in Experimental Fluid Mechanics*, M. Gad-El-Hak, Ed. (Springer, Berlin, 1989), pp. 429–456.
12. A. L. R. Thomas, G. K. Taylor, R. B. Srygley, R. L. Nudds, R. J. Bomphrey, *J. Exp. Biol.* **207**, 4299 (2004).
13. C. van den Berg, C. P. Ellington, *Philos. Trans. R. Soc. London Ser. B* **352**, 329 (1997).
14. R. J. Bomphrey, N. J. Lawson, N. J. Harding, G. K. Taylor, A. L. R. Thomas, *J. Exp. Biol.* **208**, 1079 (2005).
15. M. H. Dickinson, K. G. Gotz, *J. Exp. Biol.* **174**, 45 (1993).
16. A. Willmott, C. Ellington, *J. Exp. Biol.* **200**, 2693 (1997).
17. C. Somps, M. Luttges, *Science* **228**, 1326 (1985).
18. U. M. Norberg, in *Swimming and Flying in Nature*, vol. 2, T. Y.-T. Wu, C. J. Brokaw, C. Brennen, Eds. (Plenum, New York, 1975), pp. 869–881.
19. A. Hedenström *et al.*, *Science* **316**, 894 (2007).
20. D. R. Warrick, B. W. Tobalske, D. R. Powers, *Nature* **435**, 1094 (2005).
21. J. J. Videler, E. J. Stamhuis, G. D. E. Povel, *Science* **306**, 1960 (2004).
22. See supporting material on *Science* Online.
23. $Re = \overline{U}_{\mathrm{eff}}\overline{c}/\nu$ ($\overline{U}_{\mathrm{eff}}$ is average effective wing speed, which is the sum of the flight velocity and the wing-flapping velocity; $\overline{c}$ is the average wing chord length; and ν is the kinematic viscosity of air). *Re* is the ratio between inertial and viscous aerodynamic forces and is an index of the relative instability of the fluid around an airfoil. The bats operate at a *Re* range with rather stable aerodynamic characteristics, just below the *Re* range ($10^4 \leq Re \leq 10^5$) where the aerodynamics are notoriously hard to predict and control.
24. $St = fA/U_{\infty}$ (f is wingbeat frequency and A is the tip-to-tip vertical excursion of the wing tip). *St* is proportional to the ratio of the average wingbeat velocity to the steady forward speed and is an indication of the unsteadiness and efficiency of vortex generation.
25. For an incompressible fluid, the divergence in a planar velocity field is related to the change in out-of-plane flow velocity. When the divergence is positive, the fluid works as a fluid source in the planar velocity field, decreasing the out-of-plane velocity. When it is negative, it is a fluid sink, which increases the out-of-plane velocity.
26. E. V. Laitone, *Exp. Fluids* **23**, 405 (1997).
27. J. M. Birch, M. H. Dickinson, *Nature* **412**, 729 (2001).
28. J. M. Birch, W. B. Dickson, M. H. Dickinson, *J. Exp. Biol.* **207**, 1063 (2004).
29. J. D. Anderson, *Fundamentals of Aerodynamics* (McGraw-Hill, Singapore, 1991).
30. R. Bomphrey, N. Lawson, G. Taylor, A. Thomas, *Exp. Fluids* **40**, 546 (2006).
31. D. A. Read, F. S. Hover, M. S. Triantafyllou, *J. Fluids Struct.* **17**, 163 (2003).
32. S. M. Swartz, M. S. Groves, H. D. Kim, W. R. Walsh, *J. Zool.* **239**, 357 (1996).

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Supporting Online Material

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Synaptic Protein Degradation Underlies Destabilization of Retrieved Fear Memory

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Reactivated memory undergoes a rebuilding process that depends on de novo protein synthesis. This suggests that retrieval is dynamic and serves to incorporate new information into preexisting memories. However, little is known about whether or not protein degradation is involved in the reorganization of retrieved memory. We found that postsynaptic proteins were degraded in the hippocampus by polyubiquitination after retrieval of contextual fear memory. Moreover, the infusion of proteasome inhibitor into the CA1 region immediately after retrieval prevented anisomycin-induced memory impairment, as well as the extinction of fear memory. This suggests that ubiquitin- and proteasome-dependent protein degradation underlies destabilization processes after fear memory retrieval. It also provides strong evidence for the existence of reorganization processes whereby preexisting memory is disrupted by protein degradation, and updated memory is reconsolidated by protein synthesis.

Memory retrieval is a process of recalling a previously stored memory. Recently, memory retrieval has attracted much attention because it has been found that inhibition of protein synthesis before or immediately after memory retrieval impairs the previously consolidated memory (*1*–*4*). Retrieval of a consolidated memory thus returns the memory storage site to a labile state, after which new protein synthesis is required for stabilizing or reconsolidating the memory (*1*–*9*). This suggests that the retrieval of the consolidated memory is a dynamic and active process in which remodeling or reorganization of the already-formed memories occurs to incorporate new information (*2*, *3*, *6*).

Although it has attracted less attention than the gene transcription and protein synthesis model for long-lasting synaptic changes and memory stabilization, protein degradation is also critical for long-term memory (*10*–*16*). A major cellular mechanism controlling protein turnover is the ubiquitin and proteasome system, in which polyubiquitinated proteins are degraded by the multisubunit proteasome complex (*11*, *17*). A subunit of the 26*S* proteasome, S5a, which selectively binds to polyubiquitinated proteins, plays a critical role in protein degradation (*18*, *19*).

If retrieval stimuli trigger new protein synthesis for the remodeling of consolidated memory, protein degradation via the ubiquitin and proteasome system might be necessary because remodeling of synapses, which encode the memory, would be mediated by removal of existing proteins and by incorporation of new proteins (*11*). However, little is known about the protein degradation mechanism during the reorganization process after memory retrieval in vivo. We therefore investigated the involvement of the ubiquitin and proteasome system and the roles of protein degradation during the destabilization and restabilization process after fear memory retrieval.

We first performed a total protein polyubiquitination assay after fear memory retrieval

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(*20*). Polyubiquitinated proteins were purified by immobilized fusion proteins of S5a (*11*) from the crude synaptosomal membranes of the hippocampi, which were isolated after contextual fear memory retrieval (Fig. 1A). The polyubiquitinated protein expression levels found in the 15-min group and the 60-min group whose hippocampi were isolated 15 min and 60 min after the beginning time point of retrieval, respectively, increased remarkably, whereas the polyubiquitination level was little changed in the absence of retrieval (No R group) compared with "naïve" mice, which received only handling (Fig. 1B). Reconsolidation is not affected by a protein synthesis inhibitor when the retrieval duration is very short (up to 1 min of reexposure) (*2*). Thus, we examined the effect of retrieval duration on protein polyubiquitination. Anisomycin treatment after a 1-min reexposure did not affect the instances of conditioned freezing behavior after 24 hours (fig. S1, A and B). The amount of polyubiquitinated protein found in the 1-min reexposure group was comparable to that in the No R group and was significantly lower than that of the 5-min reexposure group (fig. S1, C and D). These findings indicate that protein polyubiquitination is increased specifically in response to a retrieval signal that induces protein synthesis–dependent reconsolidation.

We next asked which proteins are polyubiquitinated and degraded after fear memory retrieval in the hippocampus. It has been shown that neuronal activity induces the turnover and remodeling of several postsynaptic density (PSD) proteins that are critical for long-term potentiation (*11*, *21*). To examine whether proteins in PSD may be possible targets for degradation during retrieval, we performed immunoblot analyses with antibodies against three different PSD proteins: Shank, guanylate kinase–associated protein (GKAP), and PSD-95, by using glutathione *S*-transferase (GST) in complex with S5a (GST-S5a) in a pull-down assay. Polyubiquitinated Shank and GKAP, but not PSD-95, increased remarkably 1 hour after retrieval (Fig. 2, A and B). In the mouse hippocampus, multiple Shank bands, which indicate the products of alternative splicing with diverse sizes ranging from 120 kD to 240 kD, were observed as previously described in the rat cortex (*22*) (fig. S2). It was noteworthy that the amount of 130 kD and 160 kD endogenous Shank was decreased 1 to 2 hours after retrieval in the synaptic region (Fig. 2, C to E). This decrease may reflect the ubiquitin- and proteasome-dependent degradation process, as the time course of the increase of Shank polyubiquitination almost coincided with the time course of the reduction of the amount of endogenous Shank (Fig. 2). To elucidate this point more clearly, we infused either the proteasome inhibitor clasto-lactacystin-β-lactone (βlac) or vehicle into area CA1. We found that βlac infusion blocked the decrease of the amount of Shank at 2 hours after retrieval and kept the Shank level up to the basal amount in control animals without retrieval (fig. S3). Thus, the dynamic change in the expression level of specific PSD proteins such as Shank (Fig. 2, C to E) implies the possibility that synaptic destabilizing and restabilizing states are triggered after the memory retrieval process, as previously suggested (*1*–*3*, *6*). Furthermore, the protein synthesis during the reconsolidation may be a compensatory mechanism for protein degradation induced by the retrieval.

The increase in polyubiquitinated proteins at the synapses during fear memory retrieval raises a question: What is the physiological function of protein degradation after memory retrieval? We bilaterally administered βlac or the protein synthesis blocker anisomycin into the CA1 region immediately after the 1st retrieval (Fig. 3, A and B; retrieval 1). The percentage of freezing behavior was not significantly different among groups on retrieval 1 before the drug infusion (fig. S4A). Then, we retested the fear level (freezing behavior) 24 hours after the infusion (Fig. 3B; retrieval 2). We did not observe any difference between the vehicle control and the βlac group, whereas we

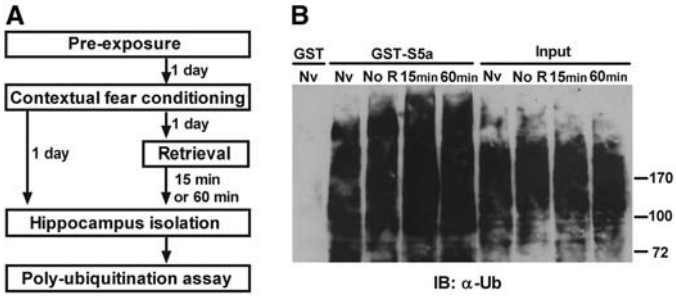

Fig. 1. Fear memory retrieval increases polyubiquitination in the hippocampal synaptosomal membrane fraction. (**A**) The schematic experimental procedure used for examining the level of polyubiquitination after retrieval. One day after contextual fear conditioning, mice were reexposed to the context to retrieve the fear memory. Then, 15 min or 60 min after retrieval, we isolated hippocampi and performed a polyubiquitination assay. (**B**) Representative data from the polyubiquitination assay show the total polyubiquitinated proteins that were pulled down by GST-S5a and detected by ubiquitin-specific antibody (α-Ub). The amount of polyubiquitination is increased 15 min and 60 min after retrieval when it is compared with the level in Nv (naïve, the mice group that received only handling) or No R (no retrieval, the mice from which hippocampi were isolated without retrieval). Polyubiquitinated proteins were not bound to GST alone. Input indicates an aliquot of total ubiquitinated proteins in each extract.

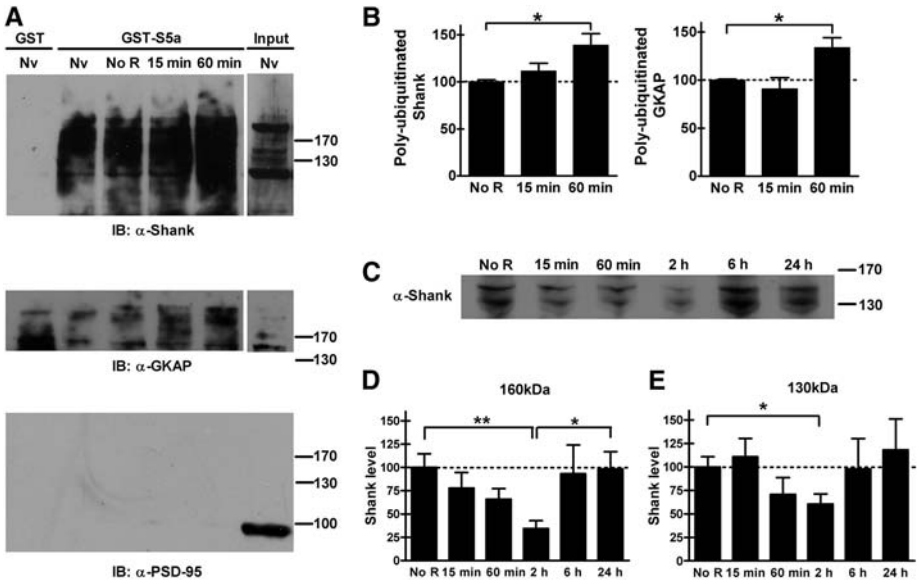

Fig. 2. Retrieval stimuli regulate the degradation of specific postsynaptic proteins. (**A** and **B**) Polyubiquitinated Shank and GKAP, but not PSD-95, are significantly increased 1 hour after retrieval. (A) Representative data show the level of Shank (top), GKAP (middle), and PSD-95 (bottom) in the isolated polyubiquitinated proteins from the hippocampal synaptosomal fraction. Polyubiquitinated proteins were isolated by the same experimental protocol as in Fig. 1. (B) Bars represent the means ± SEM of the percent change of polyubiquitinated Shank and GKAP proteins (*$P < 0.05$; unpaired *t* test; $n = 3$ for each group). (**C** to **E**) Dynamics of endogenous Shank protein after retrieval. (C) The protein expression level of Shank isoforms (160 kD, upper band; 130 kD, lower band) in the hippocampal synaptosomal membrane fraction was decreased up to 2 hours after retrieval, but it was restored to the basal level at 6 hours. (D and E) Bars represent the means ± SEM of the quantified level of Shank isoforms. Both Shank isoforms were significantly reduced at 2 hours after retrieval (*$P < 0.05$, **$P < 0.01$; unpaired *t* test; $n = 4$ for each group).

could observe fear memory impairment at retrieval 2 of the anisomycin group as reported previously (*1*, *2*) (Fig. 3, C and D). However, coinfusion of βlac with anisomycin prevented the memory impairment caused by the single infusion of anisomycin at retrieval 2 (Fig. 3, C and D). The increase in protein degradation may contribute to both the destabilization of preexisting fear memory and the restabilization of reorganized fear memory (*11*–*15*). Our data support the idea that protein degradation after memory retrieval is important for the destabilization of preexisting fear memory, rather than for the restabilization process. If the major function of protein degradation after retrieval was removal of inhibitory proteins for the memory restabilization, βlac infusion should have impaired the fear memory at retrieval 2 (*16*, *23*, *24*). However, the infusion of βlac alone did not affect the fear level. If the ubiquitin-proteasome pathway was involved in forgetting the preexisting fear memory, blocking the ubiquitin-proteasome pathway would have suppressed the anisomycin-induced impairment of fear memory retrieval by inhibiting the destruction of the preexisting fear memory (*8*). Consistent with this idea, we found that the coinfusion of βlac with anisomycin was able to prevent memory impairment caused by anisomycin after retrieval (Fig. 3, C and D). This indicates that βlac may inhibit the destruction process of the previously formed fear memory and may maintain the freezing behavior even when the reconsolidation is inhibited without new protein synthesis.

To further support this idea, we examined the effect of βlac infusion during fear memory acquisition (Fig. 4A). Because there is no preexisting fear memory at the initial phase of fear memory consolidation, the βlac infusion immediately after training may not bring about recovery from the anisomycin-induced amnesia. Consistent with this idea, we found that the infusion of βlac did not affect either anisomycin-induced memory impairment during memory acquisition or the memory acquisition itself (Fig. 4B), even though the polyubiquitinated protein level increased after fear conditioning (fig. S5). Combined with the data in Fig. 3, these results support the idea that the infusion of βlac mainly suppresses the destabilizing process of the preexisting fear memory and that protein synthesis in the hippocampus is required for the association of shock and context. The increase in polyubiquitination after conditioning may reflect the destabilization of preexisting synapses that were

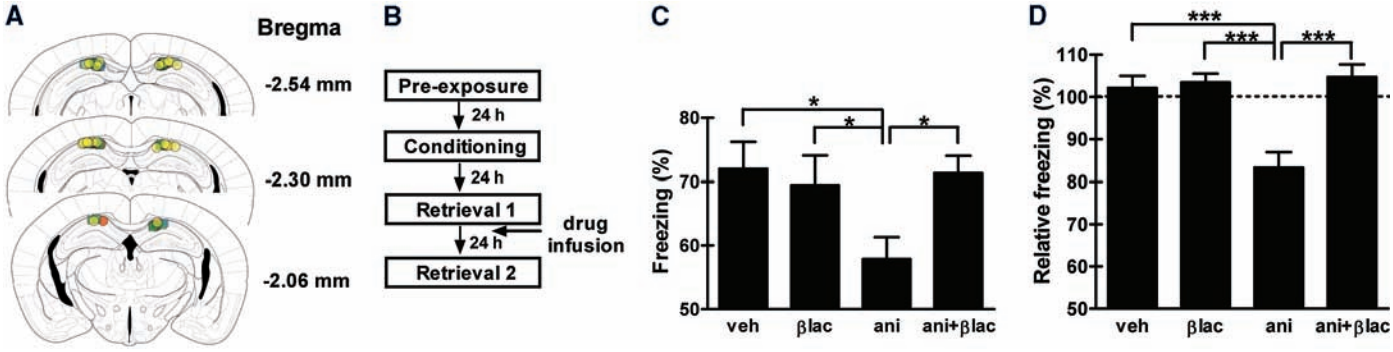

Fig. 3. Proteasome inhibitor blocks the anisomycin-induced memory impairment after retrieval. (**A**) Schematic illustration showing the cannula locations in the hippocampus at three different rostral-caudal planes. Numbers indicate the posterior direction from the bregma. Yellow circles, green squares, blue squares, and red circles indicate the infusion areas of vehicle, anisomycin, βlac, and anisomycin with βlac, respectively. (**B**) Schematic view of the experimental procedure used for evaluating the effect of βlac on the retrieval. (**C**) Bars represent the means ± SEM of the percentage of freezing behavior with respect to total number of scorings per mouse at retrieval 2. Compared with the vehicle-infused control group (veh, $n = 11$), βlac infusion (βlac, $n = 10$) alone had no effect on the fear memory retrieval, whereas the anisomycin-infused group (ani, $n = 14$) showed impaired fear memory retrieval. However, concurrent infusion of βlac with anisomycin (ani+βlac, $n = 10$) prevented the impairment of fear memory retrieval induced by anisomycin [$^*P < 0.05$; one-way analysis of variance (ANOVA) and Newman-Keuls multiple comparison test]. (**D**) Bars represent the means ± SEM of the relative freezing level of retrieval 2 [$(R_2/R_1) \times 100$ (%)]. Dotted line indicates the freezing level of retrieval 1. Significant reduction in the freezing level at the anisomycin-infused group was reversed by the coinfusion of βlac ($^{***}P < 0.001$; one-way ANOVA and Newman-Keuls multiple comparison test).

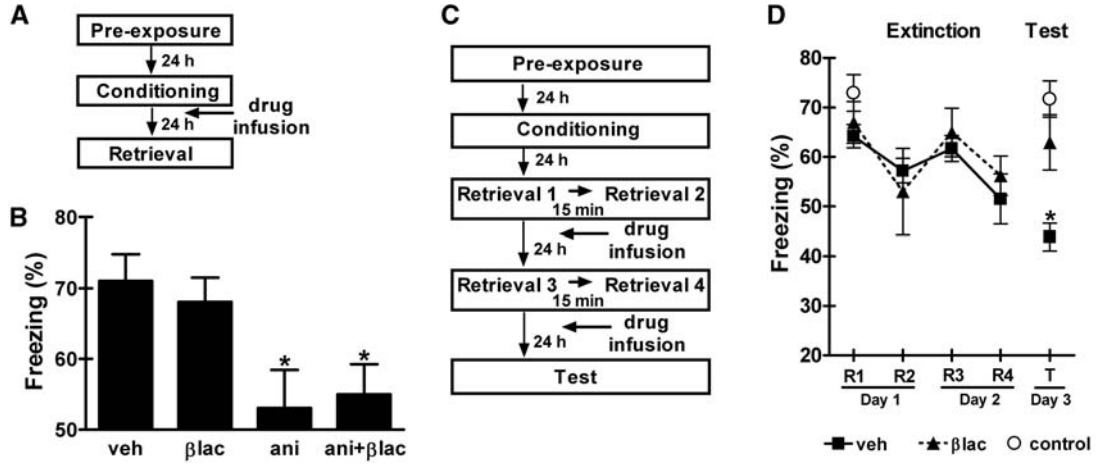

Fig. 4. Protein degradation is not required for memory consolidation, whereas it is critical for memory extinction. (**A**) Schematic view showing the protocol of behavioral experiments to examine the effect of βlac on acquiring fear memory. (**B**) Bars represent the means ± SEM of the freezing level at the retrieval day. Single infusion of βlac ($n = 12$) had no effect on the formation of fear memory compared with the vehicle-infused control group ($n = 11$), whereas the anisomycin-infused group ($n = 14$) showed significant impairment of the fear memory. Coinfusion of βlac with anisomycin ($n = 12$) also had no effect on the anisomycin-induced amnesia during memory acquisition ($^*P < 0.05$; one-way ANOVA and Newman-Keuls multiple comparison test). (**C**) Schematic view of the behavioral experiments performed to examine the effect of βlac on the fear memory extinction. (**D**) Fear memory extinction is impaired in the βlac-infused group ($n = 7$) compared with the vehicle-infused group (veh, $n = 7$). Control group ($n = 8$) indicates the vehicle-infused animals without extinction training ($^*P < 0.05$; one-way ANOVA and Newman-Keuls multiple comparison test).

related to the context or shock but did not encode the contextual fear memory.

If the retrieval induces the destabilization of the preexisting memory, it would also occur in the extinction, which is produced by repetitive retrievals in the absence of unconditioned stimuli. To test this idea, we performed the extinction training for 2 days, with two spaced retrievals per day (*25*). Either vehicle or βlac was infused immediately after the retrievals, and the freezing level was tested on the third day (Fig. 4C). It is noteworthy that βlac infusions into area CA1 suppressed the extinction of contextual fear memory (Fig. 4D and fig. S4B). The freezing behavior was significantly reduced by the extinction in the animals reexposed to the context with vehicle infusions, whereas control animals without context reexposure showed no extinction (Fig. 4D and fig. S4B). Thus, our data suggest that ubiquitin- and proteasome-dependent protein degradation is required for the memory extinction. This supports the idea that extinction is not only "inhibitory new learning" of a context–no shock association, but also involves at least some "unlearning" (or forgetting) of the preexisting context-shock association (*26*, *27*). Furthermore, extinction has been suggested to involve a memory-updating process (*27*). Combined, our results support the idea that memory retrieval makes preexisting memory labile via ubiquitin- and proteasome-dependent protein degradation in order to update or reorganize the memory with new information.

Our data also showed that infusion of βlac alone immediately after conditioning did not impair the acquisition of fear memory. This result disagrees with previous studies in some aspects (*14*, *28*). In these studies, the consolidation of inhibitory avoidance or contextual fear memory is impaired by disturbance of the ubiquitin and proteasome pathway. These discrepancies may reflect the differences in the experimental system, animal species, or brain regions involved (*29*). It is known that the circuits involved in the processing of an inhibitory avoidance task are somewhat different from the circuits of classical fear conditioning (*30*). Moreover, in contextual fear conditioning, three variables—reexposure duration, the age of the memory, and the strength of the memory—influence the memory processes activated during retrieval (*2*).

NMDA receptor activation triggers the destabilization of the consolidated fear memory (*8*). In cultured neurons, glutamatergic transmission activates the ubiquitin and proteasome system (*31*, *32*). It would be interesting to speculate that ubiquitin- and proteasome-dependent protein degradation is increased by downstream signaling of NMDA receptor activation and destabilizes the retrieved fear memory.

We have shown that ubiquitin- and proteasome-dependent degradation of preexisting postsynaptic proteins is involved in memory reorganization after retrieval. Our results support the idea that memory reorganization occurs via both degradation of preexisting synapses and synthesis of updated synapses. Preexisting memory may be rebuilt in conjunction with new information via the protein degradation and concurrent synthesis especially in the synaptic region.

References and Notes

1. K. Nader, G. E. Schafe, J. E. Le Doux, *Nature* **406**, 722 (2000).
2. A. Suzuki *et al.*, *J. Neurosci.* **24**, 4787 (2004).
3. M. H. Milekic, C. M. Alberini, *Neuron* **36**, 521 (2002).
4. P. W. Frankland *et al.*, *Learn. Mem.* **13**, 451 (2006).
5. C. M. Alberini, *Trends Neurosci.* **28**, 51 (2005).
6. R. G. Morris *et al.*, *Neuron* **50**, 479 (2006).
7. Y. Dudai, *Curr. Opin. Neurobiol.* **16**, 174 (2006).
8. C. Ben Mamou, K. Gamache, K. Nader, *Nat. Neurosci.* **9**, 1237 (2006).
9. M. Eisenberg, T. Kobilo, D. E. Berman, Y. Dudai, *Science* **301**, 1102 (2003).
10. O. Steward, E. M. Schuman, *Annu. Rev. Neurosci.* **24**, 299 (2001).
11. M. D. Ehlers, *Nat. Neurosci.* **6**, 231 (2003).
12. A. N. Hegde *et al.*, *Cell* **89**, 115 (1997).
13. A. N. Hegde, A. L. Goldberg, J. H. Schwartz, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7436 (1993).
14. M. Lopez-Salon *et al.*, *Eur. J. Neurosci.* **14**, 1820 (2001).
15. R. Fonseca, R. M. Vabulas, F. U. Hartl, T. Bonhoeffer, U. V. Nagerl, *Neuron* **52**, 239 (2006).
16. E. R. Kandel, *Science* **294**, 1030 (2001).
17. A. Ciechanover, *Nat. Rev. Mol. Cell Biol.* **6**, 79 (2005).
18. Q. Deveraux, V. Ustrell, C. Pickart, M. Rechsteiner, *J. Biol. Chem.* **269**, 7059 (1994).
19. K. Ferrell, Q. Deveraux, S. van Nocker, M. Rechsteiner, *FEBS Lett.* **381**, 143 (1996).
20. Materials and methods are available as supporting material on *Science* Online.
21. C. Luscher, R. A. Nicoll, R. C. Malenka, D. Muller, *Nat. Neurosci.* **3**, 545 (2000).
22. S. Lim *et al.*, *J. Biol. Chem.* **274**, 29510 (1999).
23. J. A. Lee *et al.*, *J. Cell Biol.* **174**, 827 (2006).
24. T. Abel, K. C. Martin, D. Bartsch, E. R. Kandel, *Science* **279**, 338 (1998).
25. K. M. Lattal, T. Abel, *J. Neurosci.* **21**, 5773 (2001).
26. K. M. Myers, M. Davis, *Neuron* **36**, 567 (2002).
27. J. Ji, S. Maren, *Hippocampus* **17**, 749 (2007).
28. Y. H. Jiang *et al.*, *Neuron* **21**, 799 (1998).
29. S. J. Martin, P. D. Grimwood, R. G. Morris, *Annu. Rev. Neurosci.* **23**, 649 (2000).
30. A. E. Wilensky, G. E. Schafe, J. E. LeDoux, *J. Neurosci.* **20**, 7059 (2000).
31. B. Bingol, E. M. Schuman, *Nature* **441**, 1144 (2006).
32. L. Guo, Y. Wang, *Neuroscience* **145**, 100 (2007).

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Hybrid Neurons in a MicroRNA Mutant Are Putative Evolutionary Intermediates in Insect CO_2 Sensory Systems

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Carbon dioxide (CO_2) elicits different olfactory behaviors across species. In *Drosophila*, neurons that detect CO_2 are located in the antenna, form connections in a ventral glomerulus in the antennal lobe, and mediate avoidance. By contrast, in the mosquito these neurons are in the maxillary palps (MPs), connect to medial sites, and promote attraction. We found in *Drosophila* that loss of a microRNA, *miR-279*, leads to formation of CO_2 neurons in the MPs. *miR-279* acts through down-regulation of the transcription factor Nerfin-1. The ectopic neurons are hybrid cells. They express CO_2 receptors and form connections characteristic of CO_2 neurons, while exhibiting wiring and receptor characteristics of MP olfactory receptor neurons (ORNs). We propose that this hybrid ORN reveals a cellular intermediate in the evolution of species-specific behaviors elicited by CO_2.

In insects, both the position of CO_2 neurons and the behavior elicited by CO_2 differ among species. For example, olfactory detection of CO_2 through neurons positioned in or around the mouthparts of an insect, such as maxillary palps (MPs) and labial palps, correlates with feeding-related behaviors. Indeed, in some blood-feeding insects such as mosquitoes and tsetse flies, these neurons are harbored in the MPs and are important in locating hosts via plumes of CO_2 that they emit (*1*–*3*). The hawkmoth, *Manduca sexta*, monitors nectar profitability of newly opened *Datura wrightii* flowers through CO_2 receptor neurons located in their labial palps (*4*, *5*). In these examples, CO_2 acts as an attractant. Conversely, in *Drosophila* CO_2 is a component of a stress-induced odor that triggers avoidance behavior (*6*). This repellent response is driven by antennal neurons expressing the CO_2 receptor complex Gr21a-Gr63a (*7*, *8*). How did these diverse behavioral responses to CO_2 arise during insect evolution? We propose that this diversity emerged through multiple steps, including changes in cellular position (arising from elimination of CO_2 neurons in one appendage and generation of these neurons in another) and changes in circuitry.