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Stepwise synaptic plasticity events drive the early phase of memory consolidation

Akihiro Goto^{1,2}, Ayaka Bota^{1,2,3}, Ken Miya^{2,4,5}, Jingbo Wang¹, Suzune Tsukamoto¹, Xinzhi Jiang¹, Daichi Hirai², Masanori Murayama^{2,6}, Tomoki Matsuda⁷, Thomas J. McHugh^{2,6}, Takeharu Nagai⁷, Yasunori Hayashi^{1,2,8,*}

Memories are initially encoded in the hippocampus but subsequently consolidated to the cortex. Although synaptic plasticity is key to these processes, its precise spatiotemporal profile remains poorly understood. Using optogenetics to selectively erase long-term potentiation (LTP) within a defined temporal window, we found that distinct phases of synaptic plasticity play differential roles. The first wave acts locally in the hippocampus to confer context specificity. The second wave, during sleep on the same day, organizes these neurons into synchronously firing assemblies. Finally, LTP in the anterior cingulate cortex during sleep on the second day is required for further stabilization of the memory. This demonstrates the precise localization, timing, and characteristic contributions of the plasticity events that underlie the early phase of memory consolidation.

The current prevailing view of episodic memory is that it is initially encoded in the hippocampus and subsequently transferred to other regions, including the cerebral cortex, for long-term storage in a process termed memory consolidation (1, 2). It has been proposed that synaptic plasticity may underlie learning, which is assumed to play a critical role in memory consolidation (3, 4). However, it remains largely unknown where and when synaptic plasticity occurs, along with the more complex question of how synaptic plasticity shapes neuronal representation. This is due primarily to a lack of appropriate experimental techniques to detect and modify synaptic plasticity in a precise spatiotemporal manner. We thus developed an optogenetic method to selectively erase long-term potentiation (LTP) without affecting basal transmission or precluding future plasticity events.

Optical erasure of sLTP

Previous studies have demonstrated that the early phase of LTP is associated with rapid polymerization of actin within dendritic spines, which acts to enlarge their structure [structural LTP (sLTP)] (5). At the same time, cofilin (CFL), an F-actin side-binding protein, accumulates at the bottom of the spine head (6). CFL exerts differential effects on F-actin de-

pending on its density on the filament (7, 8). At low density, CFL twists and severs F-actin, leading to its disassembly. By contrast, when CFL binds F-actin at a high stoichiometric ratio, it forms cofilactin, thus stabilizing F-actin. We previously demonstrated that sLTP induction promotes CFL-actin interaction, consistent with the formation of cofilactin (6). Therefore, we hypothesized that inactivating CFL would lead to destabilization of the cofilactin structure within the dendritic spine, thereby permitting selective erasure of sLTP.

To test this, we employed the genetically encoded photosensitizer protein SuperNova (SN), which allows for chromophore-assisted light inactivation (CALI) of specific molecules in living cells. Upon illumination at specific wavelengths, SN generates reactive oxygen species that inactivate the proteins to which it is fused (9, 10). In nonneuronal cells expressing a fusion protein of CFL with SN (CFL-SN), induction of CALI by light illumination inhibited the actin-dependent motility of lamellipodia, consistent with the inactivation of CFL (fig. S1) (9, 11). We then coexpressed CFL-SN with CFL-green fluorescent protein (GFP) in CA1 pyramidal neurons in hippocampal slice cultures, together with DsRed2 as a volume filler. Owing to the cooperativity of CFL binding to F-actin, we predicted that CALI of CFL-SN would also lead to dissociation of CFL-GFP from F-actin. Upon induction of sLTP by two-photon uncaging of MNI (4-methoxy-7-nitroindolyl)-glutamate at single dendritic spines, we observed a rapid accumulation of CFL-GFP, overshooting the increase in volume (6) (Fig. 1, A and B). By inducing CALI 10 min after sLTP induction, both the enrichment of CFL-GFP and the increase in spine volume were reversed.

sLTP is accompanied by decreased actin turnover within dendritic spines (12). To establish whether the enriched CFL and result-

ing cofilactin formation are involved in this process, we tested whether CALI of CFL-SN can restore actin turnover using photoactivatable GFP (PAGFP)-fused actin (12). Photoactivation of PAGFP-actin at the tip of dendritic spines revealed actin turnover within the body of the dendritic spine, which slowed after the induction of sLTP (Fig. 1C). However, when CALI was performed after sLTP induction, actin turnover was restored and spine volumes returned to baseline levels. Overall, these results are consistent with the idea that cofilactin structure maintains the increase in spine volume after sLTP induction, and that CALI of CFL-SN can efficiently reverse sLTP by destabilizing this structure and restoring actin turnover (fig. S2).

Time window of optical erasure of sLTP

To understand the temporal window of effectiveness, CALI was triggered at multiple time points after sLTP induction. Light illumination of spines expressing CFL-SN 10 min after LTP led to a decrease in spine enlargement compared with spines expressing an unfused SN (Fig. 1, D and E). Similarly, 30 min after sLTP induction, CALI remained effective in reducing spine volume. However, this effect was not evident after 50 min (Fig. 1F) (6). CALI applied to spines 1 min before sLTP had no effect on its subsequent expression (Fig. 1F). Moreover, CALI triggered in spines where sLTP had not been induced had no effect on spine volume regardless of its original size (Fig. 1, D and E, and fig. S3). Reinduction of sLTP in the same spine after CALI was still possible, indicating that the procedure does not cause permanent damage, but only temporarily disrupts the spine-associated plasticity machinery (Fig. 1E and fig. S4).

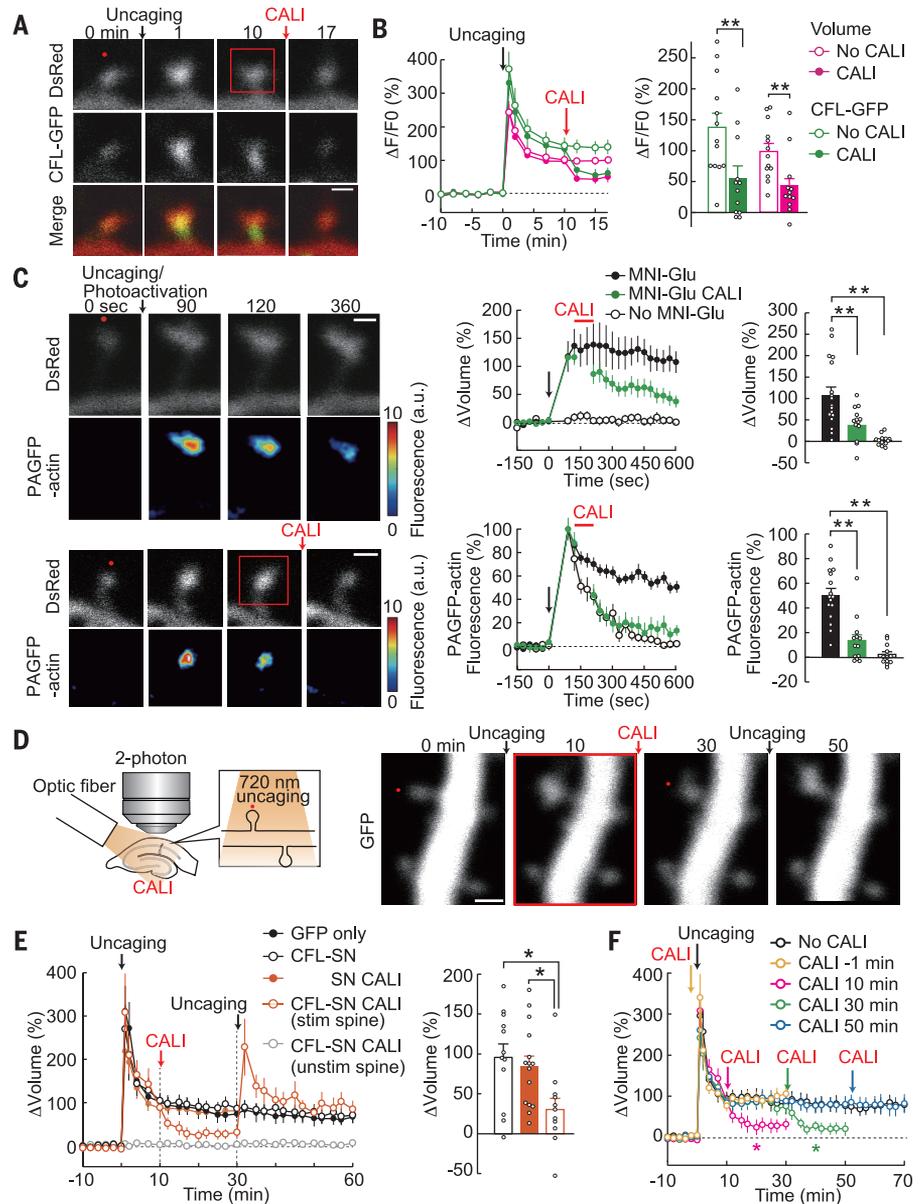
Electrically recorded LTP can also be optically erased

To test whether CALI of CFL-SN could erase electrically recorded LTP, field excitatory postsynaptic potentials (fEPSPs) were recorded in the CA1 stratum radiatum of hippocampal slices from CaMKII α -Cre mice infected with AAV₂-EF1 α -DIO-CFL-SN (fig. S5A). LTP was induced by high-frequency stimulation (HFS) and CALI was triggered 10 min later. fEPSPs were specifically decreased in the LTP pathway, but not in the control pathway or in slices expressing unfused SN (fig. S5B). Subsequent HFS produced a sustained potentiation of the fEPSPs, indicating that CALI selectively erased the existing LTP without interfering with any future plasticity events. When CALI was conducted either 1 min before or 50 min after LTP induction, it did not have any impact on potentiation (fig. S5, B and C). Because both *N*-methyl-D-aspartate receptor- and metabotropic glutamate receptor-dependent long-term depression (LTD) share CFL as a

¹Department of Pharmacology, Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan. ²RIKEN Brain Science Institute, Wako, Saitama 351-0198, Japan. ³Graduate School of Science and Engineering, Saitama University, Saitama 338-8570, Japan. ⁴Department of Molecular Neurobiology, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan. ⁵Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan. ⁶RIKEN Center for Brain Science, Wako, Saitama 351-0198, Japan. ⁷SANKEN (The Institute of Scientific and Industrial Research), Osaka University, Mihogaoka 8-1, Ibaraki, Osaka 567-0047, Japan. ⁸Brain and Body System Science Institute, Saitama University, Saitama 338-8570, Japan.

*Corresponding author. Email: yhayashi-tyk@umin.ac.jp

Fig. 1. Optical erasure of sLTP. (A) DsRed2 (top) and CFL-GFP (bottom) images. Persistent enlargement of the spine was induced with two-photon (2P) uncaging of MNI-glutamate in single dendritic spines in hippocampal slice cultures, which expressed CFL-SN, CFL-GFP, and DsRed2. The red dot in the DsRed2 image indicates the uncaging spot. A 559-nm laser was irradiated within the square region to induce CALI in the stimulated spine. Scale bar, 1 μm . (B) Time courses of changes in spine volume and the amount of CFL-GFP relative to the averaged baseline fluorescence intensity from spines without illumination ($n = 13$) and spines with 593-nm illumination 10 min after sLTP induction ($n = 12$) (left). Summary of changes in the intensity of CFL-GFP and spine volume 5 min after CALI (right). Wilcoxon signed-rank test, $P = 0.0098$ (volume), $P = 0.0043$ (CFL-GFP). (C) Images of DsRed2 and PAGFP-actin in spines. Red dots in the DsRed2 images indicate uncaging and photoactivation points. 559-nm light was illuminated within the square region around the spine head. The fluorescence of PAGFP after photoactivation was normalized to 100%. With MNI-glutamate without CALI ($n = 15$), with MNI-glutamate and CALI ($n = 14$), without MNI-glutamate and CALI ($n = 13$). Averaged spine volume 600 s after uncaging and photoactivation are shown as bar graphs. One-way analysis of variance (ANOVA) test followed by Tukey-Kramer post hoc test (versus MNI-Glu). ΔVolume ; $P = 0.0027$ (MNI-Glu CALI), $P = 0$ (No MNI-Glu), $F_{2,41} = 15.05$. Fluorescence; $P = 0.0001$ (MNI-Glu CALI), $P = 0$ (No MNI-Glu), $F_{2,41} = 17.72$. Scale bars, 1 μm . a.u., arbitrary units. (D) Field illumination of neurons expressing CFL-SN and GFP in hippocampal slice cultures (left). To induce CALI, a 593-nm laser (0.8W/cm², 60 sec, 2-mm diameter spot) was illuminated using an optic fiber. GFP images of a representative neuron expressing CFL-SN and GFP (right). Red dots indicate two-photon uncaging spots. A 593-nm laser was irradiated 10 min after LTP induction. Scale bar, 1 μm . (E) Summary of effect of CALI of CFL-SN on sLTP. Spine volume was quantified by measuring the total GFP fluorescence intensity relative to the baseline intensity. Neurons expressing GFP only without CALI (GFP only, $n = 12$), CFL-SN and GFP without CALI (CFL-SN No CALI, $n = 12$), SN and GFP with CALI (SN CALI, $n = 15$), CFL-SN and GFP with CALI (CFL-SN CALI, both glutamate stimulated and unstimulated adjacent spines, $n = 12$). Averaged spine volume 7 min after CALI are shown as bar graphs. One-way ANOVA test followed by Tukey-Kramer post hoc test. $P = 0.0119$ (CFL-SN versus CFL-SN CALI), $P = 0.0335$ (SN CALI versus CFL-SN CALI), $F_{2,36} = 5.34$. (F) Effect of CALI of CFL-SN on sLTP at various time points before and after induction. CALI was conducted 1 min before sLTP induction or 10, 30, and 50 min after induction. Wilcoxon signed-rank test, 10 min after CALI of each CALI group versus control (No CALI), $P = 0.1811$ (CALI 1 min, $n = 12$), $P = 0.0221$ (CALI 10 min, $n = 12$), $P = 0.0306$ (CALI 30 min, $n = 10$), $P = 0.9732$ (CALI 50 min, $n = 13$). Means \pm SEMs are shown; significance is indicated in the figures as follows: *: $P < 0.05$; **: $P < 0.01$.



common downstream mediator (13, 14), it was necessary to characterize the effects of CALI on LTD. However, we observed no effect on low-frequency stimulation-induced LTD (fig. S5D).

Optical erasure of LTP impairs context-specific memory within a specific time window

Next, we tested whether we could erase memories in intact animals. CFL-SN was expressed bilaterally in dorsal CA1 of the hippocampus by injecting AAV₂-EF1 α -DIO-CFL-SN in

CaMKII α -Cre mice, and optic fibers were implanted above the area of injection (Fig. 2A). Memory was assessed using an inhibitory avoidance (IA) learning paradigm. In this task, mice were placed in the lit side of a partitioned chamber. They typically crossed over to the dark side within 30 s of the door opening, where they received a foot shock (Fig. 2B). Although naïve animals (no virus injection or illumination) showed a prolonged crossover latency to the dark side on day 2, mice express-

ing CFL-SN that underwent CALI 2 min after the shock had significantly shorter latencies, indicating that memory formation was disrupted (Fig. 2C). The same mice were able to form the memory after they were shocked on day 2 without CALI and tested on day 3, ruling out any nonspecific interference with neuronal function. Animals expressing either CFL-GFP or unfused SN formed memories as normal even in the presence of illumination. Likewise, animals expressing CFL-SN

in the absence of illumination also formed a robust memory, ruling out the possibility that overexpression of CFL-SN contributes to the observed effects.

CALI was triggered at various time points after the shock. Memory was significantly impaired when CALI was conducted within 20 min after the shock (Fig. 2D). However, memory was not impaired when the light was delivered either 1 min before the animals were placed in the IA chamber or 1 hour after the shock.

To test the context specificity of the impaired memory, we prepared two IA contexts that differed in size, floor texture, visual cues, illumination color, and odor (Fig. 2E). Mice were first trained in context A without CALI. Two hours later, mice were placed in context B where they displayed a short crossover latency, indicating that they could sufficiently distinguish context B from context A (Fig. 2E). After crossover, the mice were shocked and CALI was subsequently conducted. The next day, when returned to context B, mice that underwent CALI displayed shorter crossover latencies, indicating that the memory for

context B was erased. However, when placed in context A, the same mice had crossover latencies similar to those of the control mice that did not receive CALI, demonstrating that context-specific memories can be selectively impaired.

Optical erasure of LTP during sleep also impairs memory

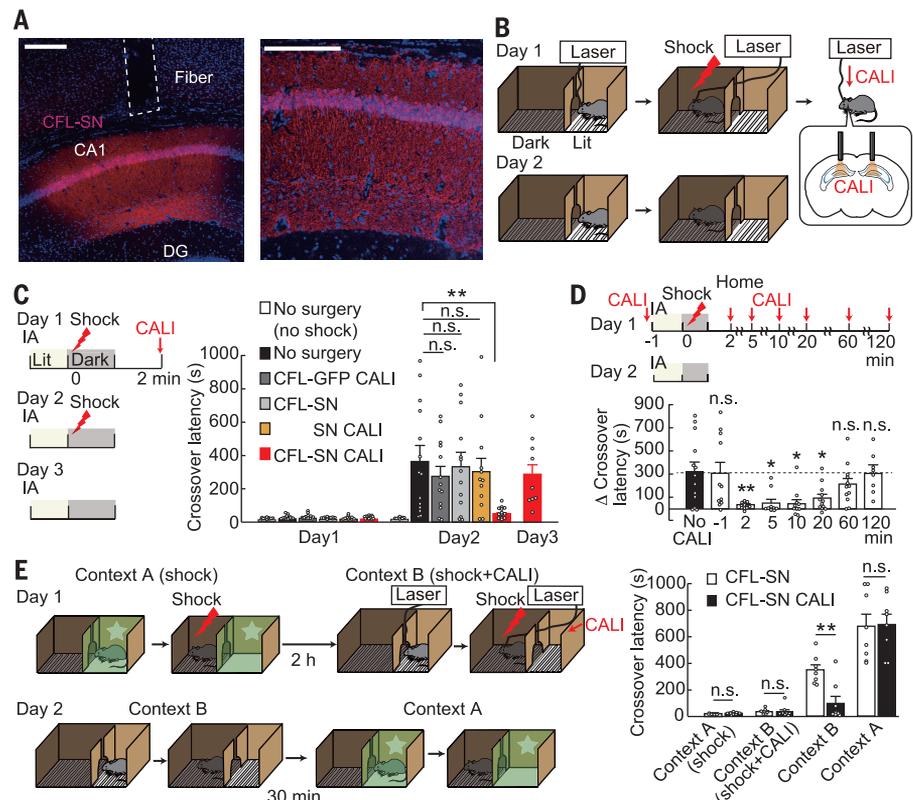
Hippocampal neuronal activity patterns associated with memory formation are subsequently replayed offline while animals are stationary or asleep, a process thought to underlie memory consolidation (15–20). However, it remains unknown whether such activity induces additional LTP in the brain; if it does, it is also unknown when and where this occurs. To establish whether additional LTP is induced offline (offline LTP) locally in the hippocampus during extended periods after learning, we illuminated the hippocampus every 20 min (the temporal resolution of the CFL-SN system) after the mice were returned to the home cage, starting 2 hours after the shock and continuing for 8 hours. We found that the memory was totally erased (Fig. 3A).

When the same group of mice were shocked on day 2 and tested on day 3, the animals exhibited normal memory, ruling out nonspecific tissue damage. To further narrow down the time window of LTP, light was delivered either in the first or second 4-hour period. The memory was still erased, though to a lesser extent in both time windows. Because local hippocampal LTP was still contributing to memory formation in the home cage up to 8 hours after the shock, we next tested whether offline LTP extends over days. We illuminated the hippocampus only on day 2, but observed no degradation of memory (Fig. 3B), suggesting that offline LTP extends more than 2 hours after learning, but consolidation is restricted to a single day locally within the hippocampus.

Hippocampal replay occurs during both wakefulness and sleep, and it has been suggested that events in these states may serve differential roles (19). We analyzed the state-dependent contribution of these processes relative to the memory consolidation process. electroencephalography (EEG) and electromyography (EMG) data were analyzed online

Fig. 2. Optical erasure of memory by CALI.

(A) Distribution of CFL-SN expressed by AAV vector in dorsal CA1 pyramidal neurons (immunostained); the optical fiber tract is indicated by a dashed line in the cortex. An AAV viral vector carrying EF1 α -DIO-CFL-SN was injected bilaterally into the dorsal CA1 of CaMKII α -Cre transgenic mice. DG, Dentate gyrus. Scale bars, 300 μ m. **(B)** Experimental protocol for inhibitory avoidance testing. **(C)** Erasure of memory by CALI of CFL-SN. Bar graph shows the average crossover latency in inhibitory avoidance test. Mice without virus injection or shock (no shock, $n = 10$), mice without virus injection but shocked (No surgery, $n = 14$), mice expressing CFL-GFP, shocked and illuminated (CFL-GFP CALI, $n = 13$), mice expressing CFL-SN, shocked but not illuminated (CFL-SN, $n = 12$), mice expressing SN, shocked and illuminated (SN CALI, $n = 12$), mice expressing CFL-SN and illuminated (CFL-SN CALI, $n = 13$). A subset of the last group was shocked again on day 2 but without illumination and tested on day 3 (CFL-SN CALI, $n = 9$). All groups except the no shock group on day 2 were statistically analyzed using one-way ANOVA tests followed by Tukey-Kramer post hoc tests (versus no surgery). $P = 0.885$ (CFL-GFP CALI), $P = 0.998$ (CFL-SN), $P = 0.986$ (SN CALI), $P = 0.0156$ (CFL-SN CALI), $F_{4,60} = 3.26$. **(D)** Time-window of the effect of CALI of CFL-SN. CALI of CFL-SN was carried out at various time points before and after shock. Mice without CALI ($n = 10$), CALI 1 min before shock ($n = 10$), CALI 2 min ($n = 10$), 5 min ($n = 10$), 10 min ($n = 10$), 20 min ($n = 10$), 60 min ($n = 10$), and 120 min ($n = 10$) after shock. One-way ANOVA test followed by Tukey-Kramer post hoc test (versus noCALI group). $P = 1$, $P = 0.0042$, $P = 0.0171$, $P = 0.0103$, $P = 0.0478$, $P = 0.797$, $P = 1$ (the order is the same as in the figure), $F_{7,82} = 5.36$. **(E)** Context selectivity of the effect of CALI. The IA test was carried out in two distinct contexts: A and B. CALI was induced 2 min after shock in context B but not in context A. Summary of crossover latency in each context with CALI ($n = 8$) and without CALI ($n = 8$). Wilcoxon signed-rank test, $P = 0.1105$ (Context A+shock), $P = 0.314$ (Context B+shock+CALI), $P = 0.004$ (Context B), $P = 0.9591$ (Context A). Means \pm SEMs are shown; significance is indicated in the figures as follows: *: $P < 0.05$; **: $P < 0.01$; n.s., not significant.



to automatically determine the behavioral state of the animal (21), and CALI was triggered separately during either awake or sleep periods that extended for at least 20 min (Fig. 3C and fig. S6). When CALI was conducted during sleep, memory was impaired (Fig. 3D). However, when it was restricted to awake periods, no impact was evident. Further, when CALI was specifically performed during sleep periods but only on day 2, it no longer impaired memory formation (Fig. 3D). Our intervention used a similar total number of illuminations across conditions (Fig. 3E) and did not alter the patterns or periods of the sleep and wake states (fig. S7).

Differential roles of online and offline LTP on the formation of hippocampal representation

So far we have demonstrated that two forms of hippocampal LTP are required for memory formation: online LTP that takes place during or immediately after the event and offline LTP that takes place during the subsequent sleep period. We next sought to establish whether these two forms of LTP have differential impacts on hippocampal representations. We imaged Ca^{2+} -responses in hippocampal excitatory neurons of active mice using a head-mounted miniaturized fluorescence microscope (22). AAV₉-CAG-DIO-CFL-SN-P2A-GCaMP6f was injected into the dorsal hippocampus of CaMKII α -Cre mice, after which a gradient index (GRIN) lens was implanted directly above

the injection site (Fig. 4A). On day 1, neuronal activity was recorded when the mice were exposed to the IA chamber without shock (Fig. 4B). On day 2, mice were reexposed to the same chamber and shocked after entering the dark side. In one group of mice, light was illuminated through the lens 2 min after the shock to erase online LTP (online CALI group). To limit the effect on the local circuit, we induced CALI unilaterally on the observed side (fig. S8). In a second group, light was illuminated every 20 min—starting 2 hours after the IA test for a total of 8 hours—to erase offline LTP (offline CALI group), whereas control groups were shocked but did not receive illumination (shock noCALI group). On day 3, all three groups were reexposed to the IA test chamber and neuronal activity was recorded. We compared the firing of individual neurons in the habituation chamber and in the IA test chamber by defining a selectivity score for each cell (see methods; Fig. 4, C to E; and fig. S9A).

CALI neurons in animals that did not receive the shock showed modest selectivity for the test chamber compared with the habituation chamber at similar levels on both day 1 and 2 (fig. S9 and Fig. 4D). By contrast, in animals that received shock (shock noCALI group), neurons fired significantly more in the test chamber than in the habituation chamber on day 3 compared with day 1, resulting in an increase in overall selectivity (fig. S9 and Fig. 4, C to E). When online LTP was erased 2 min after the

shock (shock+online CALI group), increased selectivity failed to emerge. By contrast, erasure of offline LTP did not impair the increased selectivity (shock+offline CALI group).

To dissect the impact of offline LTP on the hippocampal synaptic circuit, we analyzed the population Ca^{2+} dynamics using principal component analysis (PCA) (23) (Fig. 4F). In the shock-only mice, after IA learning we observed that activity repeatedly deviated from the trajectories of day 1. In epochs where these deviations were observed, Ca^{2+} signals increased in multiple cells shared between the epochs (Fig. 4G), indicating that they reflect the recurring synchronous activity of specific sets of neurons. We confirmed that synchronous events occurred primarily when the deviations were observed and that the number of synchronous events increased after the shock, though the mean firing rate did not change between days 1 and 3 (Fig. 4H). The deviation in PCA, as well as the increase in synchronous events, were not evident when either online LTP or offline LTP were erased (Fig. 4, F and H, and fig. S10C), indicating the importance of both forms of LTP for synchronous activity after learning. Before door opening on day 3, synchronous firing was observed broadly around the center of the chamber, whereas after door opening, it was most pronounced adjacent to the door (Fig. 4I and fig. S10, A and B). This suggests that such assembly activity may reflect recall of the training episode. CALI eliminated such

Fig. 3. Offline LTP in the hippocampus during sleep is required for memory.

(A) Repeated CALI in the home cage erased memory. Light was delivered while animals were in the home cage every 20 min for 8 hours, either 2 hours after shock ($n = 8$), the first 4 hours ($n = 10$), or the second 4 hours ($n = 9$). Animals only expressing SN were illuminated and used as a control ($n = 9$). Animals received 8 hour repeated illumination, and shock was given again on day 2 without CALI; memory was then retested on day 3 ($n = 8$). One-way ANOVA test followed by the Tukey-Kramer post hoc test (versus SN CALI), $P = 0.0079$ (0 to 8 h), $P = 0.0425$ (0 to 4 h), $P = 0.1153$ (4 to 8 h), $F_{3,32} = 4.53$. (B) Same as in (A) except that CALI was conducted the next day and memory was tested on day 3 (CFLSN-CALI, $n = 11$). Control mice did not express CFL-SN (No virus, $n = 10$). Wilcoxon signed-rank test, $P = 0.843$. (C) Automatic detection of behavioral state. EEG and EMG were recorded and analyzed online using a fast Fourier transform (FFT) every 4 sec. Light was illuminated during sleep or wake periods lasting for ≥ 20 min. Examples of sleep states and light illumination (red line) are shown on the right. (D) CALI of CFL-SN erases memory during sleep on the same day, but not the next day. Light was illuminated either during sleep (Sleep, $n = 9$) or wake (Awake, $n = 8$) periods commencing 2 hours after shock, and mice were returned to the IA box again on day 2. Control mice did not undergo CALI (No virus, $n = 9$). The experimental group (Sleep-day 2, $n = 10$) received light during sleep on the next day and were returned to the IA box on day 3. One-way ANOVA test followed by Tukey-Kramer post hoc test (versus Sleep), $P = 0.0135$ (No virus), $P = 0.0251$ (Awake), $F_{3,32} = 4.59$. (E) Average number of CALI was not different among Sleep, Awake, and Sleep-day 2 groups. One-way ANOVA test, $P = 0.1449$. Means \pm SEMs are shown; significance is indicated in the figures as follows: *, $P < 0.05$; **, $P < 0.01$; n.s., not significant.

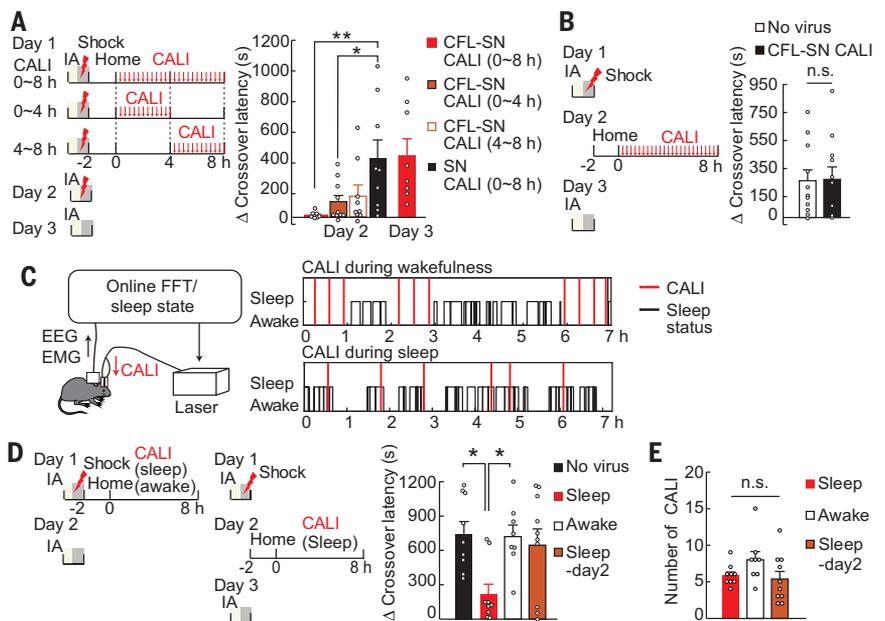
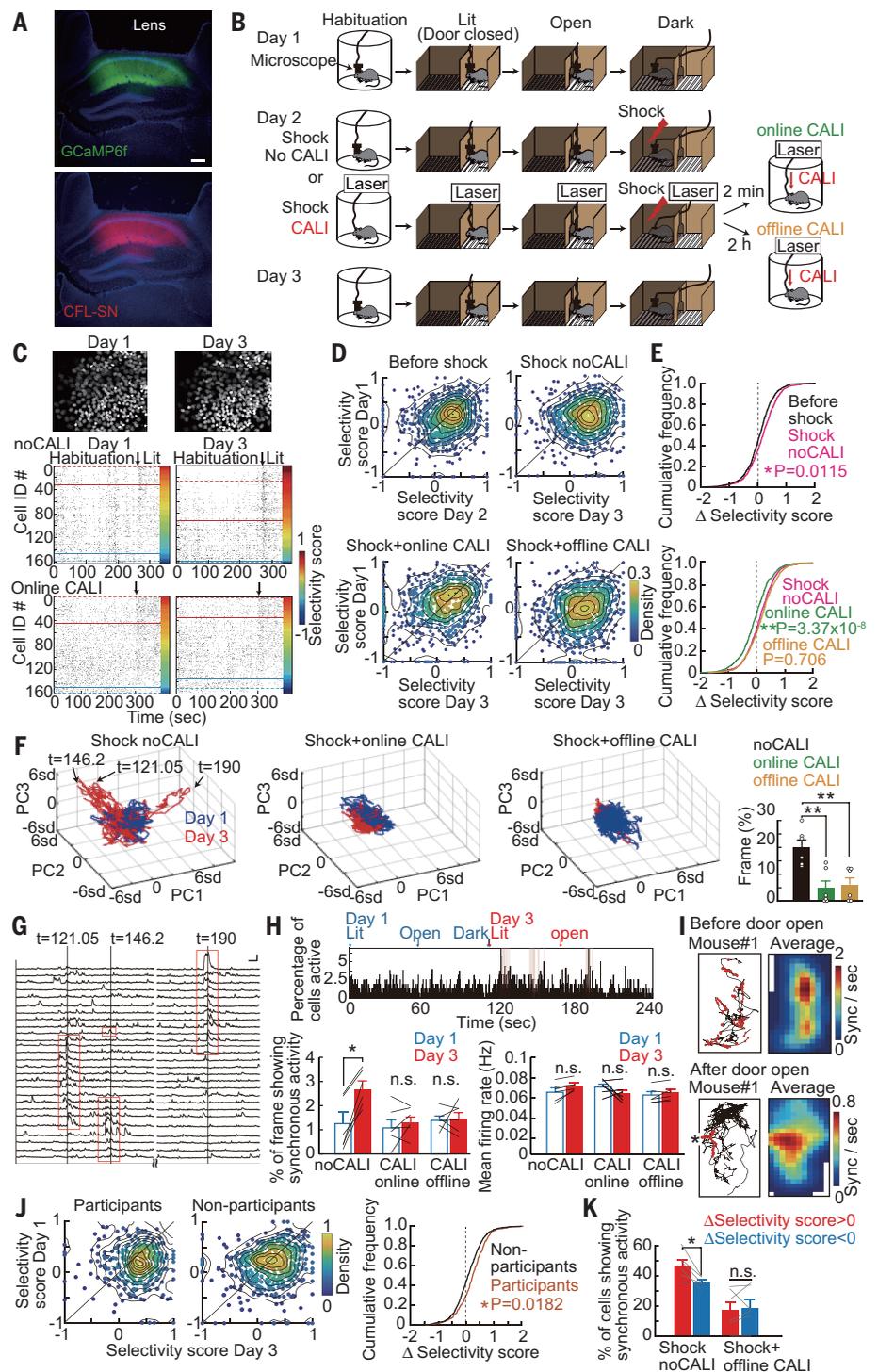


Fig. 4. Hippocampal online LTP and offline LTP have distinct roles in memory engram formation.

(A) Expression of CFL-SN and GCaMP6f by AAV₉-CAG-DIO-CFL-SN-P2A-GCaMP6f in CA1 pyramidal neurons. **(B)** Experimental protocol of Ca²⁺ imaging during IA testing and CALI. **(C)** Images of detected cells on day 1 (before shock) and on day 3 (after shock) from a Shock noCALI mouse. Raster plot of representative cells commonly active on day 1 and day 3. Ca²⁺ events (>2 events) are plotted per sec and cells are sorted in order of selectivity score on each day. Red and blue horizontal lines indicate selectivity scores of 0.4 and -0.4, and dotted lines indicate scores of 0.8 and -0.8. Shock noCALI (top) and Shock+online CALI (bottom) mice. Raw values are shown in fig. S9B. **(D)** Selectivity score of cells on day 1 versus day 2 (Before shock, 852 cells, *n* = 6 mice) or on day 1 versus day 3 (Shock noCALI, 776 cells, *n* = 6 mice, Shock+CALI online, 763 cells, *n* = 6 mice, Shock+CALI offline, 935 cells, *n* = 6 mice) are shown. Density of plot is displayed in pseudocolor, with contours depicted as black lines. **(E)** Same data as in (D) but differences in selectivity scores are plotted in cumulative histograms. Kolmogorov-Smirnov test: No shock versus Shock noCALI (top), and with respect to control (Shock noCALI) (bottom). **(F)** Principal component analysis (PCA) of concatenated Ca²⁺ traces of day 1 and day 3 data from a mouse shocked on day 2 without CALI (left). Population trajectories were projected onto the first three principal components. Arrows indicate time points with high deviation on day 3. 6sd denotes 6 times standard deviation (SD) of PCA score on day 1. Similar PCA analysis from a mouse shocked on day 2 that underwent CALI 2 min after shock to cancel online LTP (Shock+online CALI, middle), and a mouse shocked on day 2 that underwent CALI in the home cage to cancel offline LTP (Shock+offline CALI, right). The bar graph displays the average percentage of frames that showed PCA scores on day 3 that had >3 SD of the PCA score on day 1. One-way ANOVA test followed by Tukey-Kramer post hoc test (versus Shock noCALI, *n* = 6 mice), *P* = 0.0028 (Shock+online CALI, *n* = 6 mice), *P* = 0.0058 (Shock+offline CALI, *n* = 6 mice), *F*_{2,15} = 10.04. **(G)** Sample traces of neurons from a Shock noCALI mouse in (F). (*t* = 121.05, 146.2, and 190). Red rectangles indicate activation at those time points. Scale: 50% ΔF/F, 5 s. **(H)** Percentage of cells active on day 1 and day 3 (top). The red area indicates frames where high deviation was observed in (F). Percent of frame showing synchronous activity (bottom left) and mean firing rate (bottom right) on day 1 and day 3. Paired *t*-test, **P* < 0.05. (noCALI, *n* = 6 mice, CALI 2 min, *n* = 6 mice, CALI Sleep, *n* = 6 mice). **(I)** Synchronous firing is selectively seen near the door to the dark side. Trajectory (black line) and location where high deviation was observed in PCA analysis (red dots) from a representative shock-only mouse on day 3, before and after the door opened (left). Heat map of average synchronous activity rate in lit chamber on day 3 (right). The number of synchronous activities detected by PCA analysis in each bin was pooled from 6 mice and divided by total their occupancy time in each bin. The asterisk indicates door location. **(J)** Selectivity score from cells that participated in synchronous activity were increased after shock, but no increase was observed for those that did not participate in synchronous activity. Cells that were activated (>4 SD of basal Ca²⁺ signal) at least once in the frame when high deviation was observed by PCA analysis were classified as participants. (Participant, *n* = 309, nonparticipant *n* = 467 from 6 mice). Kolmogorov-Smirnov test. **(K)** Percent of cells showing synchronous activity in the group of cells showing a higher selectivity score after shock (red bar, cells below the diagonal line in Fig. 4D) and that in the group of cells showing lower selectivity score after shock (blue bar, cells beyond the diagonal line). Paired *t*-test, *P* = 0.014, *n* = 6 mice (noCALI), *P* = 0.669, *n* = 6 mice (offline CALI). Means ± SEMs are shown; significance is indicated in the figures as follows: **P* < 0.05; ***P* < 0.01; n.s., not significant.



accumulation (fig. S10, A and B). We then classified neurons in the noCALI group into two classes based on their participation in synchronous events using PCA analysis, then separately calculated selectivity scores (Fig. 4J). Cells that participated in synchronous activity at least once displayed a higher selectivity score after learning (day 3) than before (day 1), although those that did not exhibited a significant reduction in the degree to which the selectivity score was modulated by learning. We then classified the cells into two groups based on the changes in selectivity score before and after shock (Fig. 4K). In noCALI animals, cells that showed an increase in selectivity score after shock were more likely to participate in synchronous activity than those that showed a decrease. In offline CALI animals, the fraction of cells participating in synchronous activity was overall lower than that of noCALI animals and was comparable between cells showing an increase in selectivity score after shock and those showing a decrease. These results are indicative of a causal relationship between selectivity score and synchronous activity, which can be erased by offline CALI.

Erasure of LTP in ACC during sleep on the following day impairs memory

Finally, we attempted to better understand the process of memory transfer to the cortex by focusing on the anterior cingulate cortex (ACC), which is activated during recall of remote memory (24–26). CFL-SN was expressed in ACC excitatory neurons using AAV₂-EF1 α -DIO-CFL-SN in CaMKII α -Cre mice, and optical fibers were bilaterally implanted above ACC (Fig. 5). First, we explored the time window of synaptic plasticity within the ACC. In contrast to CALI in the hippocampus, CALI in the ACC that was triggered either 2 min after shock (Fig. 5) or every 20 min for 8 hours (commencing 2 hours after shock) did not impair memory expression (Fig. 5). By contrast, when CALI was induced every 20 min for 8 hours on day 2 and memory was assessed on day 3, we observed a robust erasure of the memory (Fig. 5). However, this was not the case when the manipulation was performed on day 25 (Fig. 5). When illumination in the ACC was restricted to either sleep or awake periods on day 2, memory could be effectively erased only during sleep (Fig. 5), demonstrating that plasticity in the ACC occurs 1 day after learning and most likely reflects the mechanism by which memories are transferred from the hippocampus to the ACC.

We developed a versatile optogenetic method allowing for the selective optical erasure of LTP in a spatially and temporally restricted manner. It effectively erases established LTP, without altering basal transmission or interfering with future LTP. This is different from other genetic or pharmacological approaches,

where temporal or cell-type specificities are difficult to attain (27, 28). Although other tools exist that can erase LTP, such as AS-PARac, PA-AIP, and eosin-tagged AMPA receptor antibody (29–31), our method is able to erase the early phase of LTP. This differs from AS-PARac, which is aimed at the late protein synthesis phase of LTP, as it has a much wider temporal window of intervention than PA-AIP (~1 min). It is also purely genetically encoded, unlike the eosin-tagged antibody, making it an effective method for in vivo manipulation of memories.

We found that hippocampal LTP occurs as two distinct temporal processes: online immediately after learning and offline during periods of sleep. These two processes have distinct roles: Online LTP establishes the selectivity of neuronal firing to the shock context, as previously reported (32), and offline LTP is predominantly responsible for the recruitment of those neurons into repeated bouts of synchronized firing. Synchronized activity was observed when animals looked into the dark side of the chamber but did not enter, possibly reflecting recall of the shock context. Our results indicate that this synchronous activity induces further LTP in neurons, which serves to stabilize the nascent memory engram encoding the abstract features encompassing an episode.

After the two waves of LTP in the hippocampus, a third wave of extra-hippocampal LTP takes place during sleep the next day in the

ACC; this is required for systems consolidation. On the other hand, LTP in the hippocampus is no longer required for memory recall. Our data are consistent with a recent study that demonstrated the rapid generation of immature engram cells after training in the prefrontal cortex (33). They proposed that a memory engram can be formed as early as after 1 day in the ACC, but remains silent. Subsequent consolidation is required to become a fully mature engram (2, 33). The density of dendritic spines consistently remains unchanged in ACC after one day but subsequently increases, possibly reflecting the maturation process, and is required for memory consolidation (34). In our study, the reversal of ACC plasticity on day 2 had already impaired memory 24 hours later, suggesting that even at this early point in systems consolidation, cortical circuits can play a role in memory recall. The discrepancy between our study and that of Kitamura *et al.* (33) is not clear at this point, but it may be due to differences in the method of inactivation (tetanus toxin to block output versus CFL-SN to erase LTP while leaving basal activity intact).

Synaptic plasticity in ACC during sleep is also most likely mediated by replay, with the high-frequency oscillatory activity that occurs across hippocampal-cortical networks thought to be key in promoting the strengthening of synaptic connections (35). Reinforcing the coordination between hippocampal sharp wave

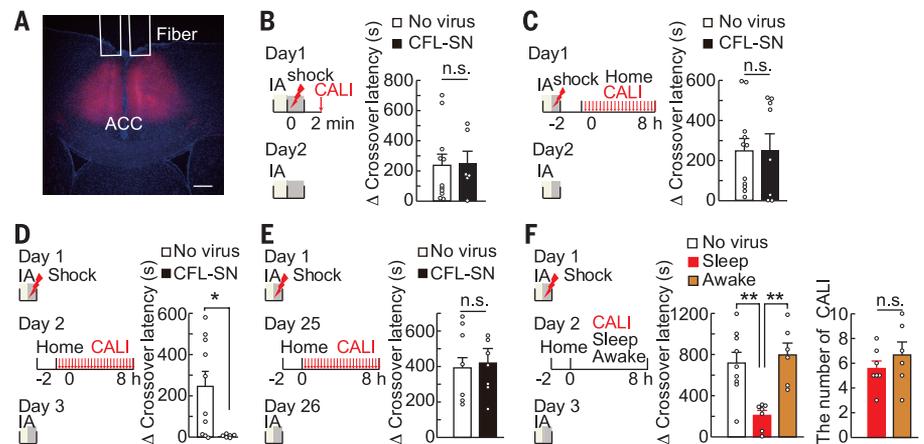


Fig. 5. ACC has an LTP time window distinct from that of the hippocampus. (A) Expression of CFL-SN in ACC neurons. Scale bar, 200 μ m. (B to E) Effect of CALI in ACC 2 after shock. (B) 2 min, (C) 2 to 8 hours, (D) 1 day, and (E) 25 days after. Memory was erased only when CALI was performed 1 day later (2 min after; No virus, $n = 11$; CFLSN-CALI, $n = 6$. 2 to 8 hours; No virus, $n = 11$; CFLSN-CALI, $n = 8$, 1 day after; No virus, $n = 10$; CFLSN-CALI, $n = 6$. 25 days after; No virus, $n = 7$; CFLSN-CALI, $n = 7$). All experiments were done during the light cycle. Wilcoxon signed-rank tests, $P = 0.81$ (B), $P = 0.90$ (C), $P = 0.025$ (D), $P = 0.70$ (E). (F) Offline LTP during sleep on next day in ACC is required for memory formation. 26 hours after shock, light was illuminated either during sleep or during wake for 8 hours. All experiments were done during the dark cycle. One-way ANOVA test followed by Tukey-Kramer post hoc test with respect to sleep ($n = 10$), $P = 0.0029$ (No virus, $n = 9$), $P = 0.0019$ (awake, $n = 8$), $F_{2,19} = 10.37$. Average number of CALI trials did not differ between sleep CALI and awake CALI groups. Wilcoxon signed-rank test, $P = 0.43$. Means \pm SEMs are shown; significance is indicated in the figures as follows: *, $P < 0.05$; **, $P < 0.01$; n.s., not significant.

ripples and cortical waves by electrical stimulation resulted in enhanced recall performance, whereas disruption of coupling resulted in memory impairment (36, 37). Likewise, disruption of replay during sleep (17) has been shown to impair performance in memory-dependent tasks; however, the exact nature of the mechanisms responsible for inducing plasticity in ACC remains to be elucidated.

In conclusion, we show clear evidence that the early phase of systems consolidation is composed of multiple steps of synaptic plasticity; presumably each undergoes synaptic consolidation. Further application of the introduced optogenetic tool, possibly with color variants of SN (38), will allow for a more comprehensive temporal dissection of synaptic plasticity events across multiple neuronal structures and populations, thus providing a much clearer picture of memory consolidation. Although this tool was not directly shown as working for sLTP in vivo associated with learning, it may enable identification of spines that undergo synaptic plasticity during memory consolidation through combination with in vivo imaging.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S10

References (39, 40)

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Stepwise synaptic plasticity events drive the early phase of memory consolidation

Akihiro GotoAyaka BotaKen MiyaJingbo WangSuzune TsukamotoXinzhi JiangDaichi HiraiMasanori MurayamaTomoki MatsudaThomas J. McHughTakeharu NagaiYasunori Hayashi

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Where and when of memory consolidation

Episodic memory is initially encoded in the hippocampus and later transferred to other brain regions for long-term storage. Synaptic plasticity underlies learning and plays a critical role in memory consolidation. However, it remains largely unknown where and when synaptic plasticity occurs and how it shapes the neuronal representation. Goto *et al.* developed a new tool for controlling early structural long-term potentiation (sLTP). By selectively manipulating sLTP, the authors showed that the local circuitry in hippocampal area CA1 is required for memory formation shortly after the encoding event. The local circuitry is also important for offline memory consolidation within 24 hours. The anterior cingulate cortex, another brain region directly connected with area CA1, is crucial for memory consolidation during sleep on the second night. —PRS

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