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Silent memory engrams as the basis for retrograde amnesia

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Recent studies identified neuronal ensembles and circuits that hold specific memory information (memory engrams). Memory engrams are retained under protein synthesis inhibition-induced retrograde amnesia. These engram cells can be activated by optogenetic stimulation for full-fledged recall, but not by stimulation using natural recall cues (thus, amnesia). We call this state of engrams “silent engrams” and the cells bearing them “silent engram cells.” The retention of memory information under amnesia suggests that the time-limited protein synthesis following learning is dispensable for memory storage, but may be necessary for effective memory retrieval processes. Here, we show that the full-fledged optogenetic recall persists at least 8 d after learning under protein synthesis inhibition-induced amnesia. This long-term retention of memory information correlates with equally persistent retention of functional engram cell-to-gram cell connectivity. Furthermore, inactivation of the connectivity of engram cell ensembles with its downstream counterparts, but not upstream ones, prevents optogenetic memory recall. Consistent with the previously reported lack of retention of augmented synaptic strength and reduced spine density in silent engram cells, optogenetic memory recall under amnesia is stimulation strength-dependent, with low-power stimulation eliciting only partial recall. Finally, the silent engram cells can be converted to active engram cells by overexpression of α-p21–activated kinase 1, which increases spine density in engram cells. These results indicate that memory information is retained in a form of silent engram under protein synthesis inhibition-induced retrograde amnesia and support the hypothesis that memory is stored as the specific connectivity between engram cells.

Significance

We previously discovered that memory is retrieved robustly from protein synthesis inhibitor-induced retrograde amnesia by optogenetic activation of engram cells. Connectivity of engram cells correlates with memory information storage under amnesia, even though these amnesic engram cells lack learning-induced augmented synaptic strength. We term this state of engrams as “silent engrams.” The significance of this study is threefold: first, the silent state of the engram can last for a prolonged period (at least 8 d post encoding); second, connectivity between engram cell ensembles is causally linked to optogenetic recall and hence memory information storage; and third, there is a molecular genetic method to convert an engram from a silent state to an active state.


The authors declare no conflict of interest.

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in DG cells (Fig. 1 A–C), as demonstrated by comparable levels of engram labeling in saline and anisomycin-treated groups. We next examined the behavioral effect of optogenetically stimulating DG engram cells in anisomycin-treated mice 2, 5, and 8 d post-CFC training (Fig. 1D). Before engram cell labeling, habituation to context A with light-off and light-on epochs did not cause freezing behavior in naïve mice of the presaline or pre-anisomycin groups (Fig. 1E). One day after training, the saline group displayed robust freezing behavior, whereas the anisomycin group showed significantly less freezing during cued recall (Fig. 1F). Two days after training, mice were placed into context A for a blue light stimulation (engram activation) test session. Consistent with our previous study (6), neither group showed freezing behavior during light-off epochs, but both groups froze significantly during light-on epochs (Fig. 1G, Left). To assay the conditioned response to natural recall cues, mice were again tested in context B 24 h after the blue light was turned off, and retrograde amnesia was observed in the anisomycin-treated group (Fig. 1G, Right).

Next, we investigated functional engram cell–engram cell connectivity between the upstream engram cell ensemble in DG and the downstream engram cell ensemble in hippocampal CA3 and, further downstream engram cell ensemble in the basolateral amygdala (BLA) by quantifying the overlap of engram cells and endogenous c-Fos+ cells in the downstream sites, that appear due to stimulation by recall cues (Fig. 1 H and I). Natural recall cues resulted in reduced c-Fos+/mCherry overlay in both CA3 and BLA of anisomycin mice, 2 d after training. However, DG engram cell activation by blue light in amnesic mice resulted in equivalent overlap as natural cue-induced recall and optogenetically induced recall in saline mice (Fig. 1J). Remarkably, the recovery from amnesia through direct light activation of anisomycin-treated DG engram cells and the preferential, protein synthesis-independent functional engram connectivity was observed both 5 d (Fig. 1 K and L) and 8 d (Fig. 1 M and N) after training. These data indicate that the connectivity of DG engram cells and the downstream CA3 and BLA engram cells is retained for as long as 8 d after training even in the anisomycin-treated animals (Fig. 1O). These results also reveal that memory engram cells can be at least of two different states: (i) the “silent” state that is not susceptible to natural recall cues for reactivation but can be reactivated by optogenetic stimulation and (ii) the active state that is susceptible to both natural and optogenetic stimuli.

**Strong Engram Stimulation Is Necessary for Memory Recall Restoration in Amnesic Mice.** Although silent memory engrams persist in mice under protein synthesis inhibitor-induced retrograde amnesia, these engram cells lack learning-induced synaptic strength (6), which is a characteristic of active, consolidated memory engram cells. We investigated the strength dependency of optogenetic stimuli in reactivating silent engram cells for recall in retrograde amnesia. For this purpose, we used three levels of blue laser power: 25, 50, and 75% (Fig. 2 A and B). Using ex vivo electrophysiology, the effect of the three levels of blue laser power on engram cell activation was validated (Fig. 2C). At 25% laser power, direct light activation of DG engram cells resulted in memory recall in saline mice, but not in anisomycin mice (Fig. 2D). Optogenetic activation of DG engram cells was comparable in saline and anisomycin mice, as demonstrated by the overlap between light-induced endogenous c-Fos+ cells and ChR2-labeled engram cells (Fig. 2E, Left). Crucially, engram activation in amnesic mice at this laser power showed significantly reduced c-Fos+/mCherry overlap in downstream CA3 and BLA engram cells, compared with the overlap in saline mice (Fig. 2E, Middle and Right). In contrast, at 50% (Fig. 2 F and G) and 75% (Fig. 2 H and I) laser power, engram activation in amnesic mice resulted in memory recall and levels of downstream engram cell reactivation equivalent to those of saline mice. Together, these data indicate that stronger optogenetic stimulation is required for memory restoration in amnesic mice (Fig. 2J), reflecting the silent state of memory engrams under this condition.

**Inactivation of the Connectivity with Downstream Engram Cell Ensembles Prevents Optogenetic Memory Recall.** The correlation between long-term (at least 8 d after encoding) retention of memory and the connectivity between upstream and downstream engram cell ensembles (2, 6) (Fig. 1) under amnesia suggests that there is a causal link between these two phenomena. To test this possibility, we developed a viral approach using tetanus toxin light chain (TetTox) (27) to inactivate engram-specific connections. We first inactivated the upstream medial entorhinal cortex (MEC) to DG engram cell connections by injecting a virus mixture of AAV9-c-fos-tTA (22) and AAV9-TRE-GFP-TetTox into MEC, while simultaneously labeling DG engram cells with a mixture of AAV9-c-fos-tTA and AAV9-TRE-ChR2-mCherry (Fig. 3 A–C). We validated learning-dependent labeling of MEC neurons with TetTox and that engram labeling in MEC and DG was comparable between saline and anisomycin groups (Fig. 3D). As expected, in natural memory recall tests of the CFC paradigm (Fig. 3 E and F), anisomycin eYFP and anisomycin TetTox mice displayed retrograde amnesia. Importantly, the effect of TetTox expression on engram cells in DG-to-CA3 was shown by reduced freezing in saline TetTox mice compared with saline eYFP mice (Fig. 3F, Right). We also observed decreased overlap of c-Fos+/mCherry DG engram cells following natural memory recall (Fig. 3F, Right) in saline TetTox mice compared with saline eYFP mice. Two days after training, although MEC-to-DG engram cell connections were inactivated, DG engram activation in both eYFP and TetTox groups of saline and amnesic mice resulted in memory recall (Fig. 3G) and downstream engram cell reactivation (Fig. 3H). By contrast, when downstream DG-to-CA3 engram cell connections were inactivated (Fig. 3 I and J), DG engram activation resulted in memory recall (Fig. 3K) as well as downstream engram cell reactivation (Fig. 3L) in saline and anisomycin eYFP groups, but not in the saline and anisomycin TetTox groups. Interestingly, the saline TetTox mice in which DG-to-CA3 connections were inactivated showed normal levels of natural memory recall. This probably indicates that the direct entorhinal cortex to hippocampal CA3 engram cell input is sufficient for memory recall even if DG-to-CA3 connections were inactivated (28). These experiments demonstrate that an inactivation of engram cell connectivity downstream, but not upstream, prevents light-induced memory recall.

**Conversion of a Silent Engram to an Active Engram by PAK1 Overexpression.** Active engram cells exhibit a learning-induced increase of dendritic spine density, which is lacking in silent engram cells (6, 22, 29). We investigated the spine density of engram cells in saline- and anisomycin-treated mice 2, 5, and 8 d post-CFC training. The anisomycin group showed reduced DG engram cell-specific spine density, compared with the saline group, across all three time points (Fig. 4 A–C). By tagging the hippocampal CA1 component of a contextual fear memory engram with ChR2-eYFP (Fig. 4 D and E), we found that anisomycin-treated CA1 engram cells showed reduced spine density compared with the saline-treated CA1 engram cells (Fig. 4 F–H). This finding is consistent with data from anisomycin-treated DG engram cells (6). We then hypothesized that the reversal of dendritic spine deficits in CA1 engram cells of anisomycin-treated mice may rescue long-term memory by natural recall cues. To investigate this possibility, we took advantage of previous findings that spine formation can be regulated by α21-activated kinase 1 (PAK1) overexpression (30, 31). We developed an adeno-associated virus (AAV) approach for the
Fig. 1. Long-term stability of functional engram cell–engram cell connectivity in retrograde amnesia. (A and B) Hippocampal DG sections from c-fos-tTA mice injected with AAV9-TRE-ChR2-eYFP virus, 24 h after saline (A) or anisomycin (B) injections. (C) Cell counts from saline (n = 4) and anisomycin (n = 5) groups. (D) Behavioral schedule. Beige shading signifies that mice were on a DOX diet, precluding ChR2-eYFP expression. Mice were habituated to optogenetic blue light stimulation at 20 Hz (~10–12 mW at patch cords). Mice were taken off DOX 24 h before CFC. Saline or anisomycin was injected immediately after training. One day after training, a natural recall test was performed (test 1). At 1, 4, or 7 d later, mice received an optogenetic recall (Engagement activation) session. The next day a natural recall (test 2) session was performed. (E) Habituation. Presaline (n = 10) or preanisomycin (n = 12) groups. (F) Natural recall (test 1). (G) Optogenetic recall (Left) 2 d posttraining, and natural recall (test 2, Right). Saline (n = 10) and anisomycin (n = 12) groups. (H and I) Cell counting 2 d posttraining using c-fos-tTA mice with AAV9-TRE-ChR2-eYFP injected into the DG and AAV9-TRE-mCherry injected into both CA3 and BLA. Following natural recall or optogenetic recall, mice were perfused 1 h later. Images showing c-Fos expression (green), mCherry engram labeling (red), and c-Fos/mCherry overlap (white circles) in CA3 (H) and BLA (I). (J) c-Fos+/mCherry+ overlap in CA3 (Left) and BLA (Right) (n = 4 per group). Chance levels, indicated by black dashed lines, were estimated at 0.88 (CA3) and 0.49 (BLA). Anisomycin natural cues group data in CA3 and BLA are significantly greater than chance level (one-sample t tests against chance for CA3 and BLA, P < 0.05). Average mCherry labeling was 13.4% (of DAPI) in CA3 and 9.4% in BLA. Average cFos was 10.1% in CA3 and 7.6% in BLA except for the anisomycin natural recall group, which was 6.1%. (K) Optogenetic recall (Left) 5 d posttraining, and natural recall (test 2, Right). Saline (n = 9) and anisomycin (n = 10) groups. (L) Cell counting 5 d posttraining. c-Fos+/mCherry+ overlap (n = 5 per group). Chance at 0.79 (CA3) and 0.41 (BLA). (M) Optogenetic recall (Left) 8 d posttraining and natural recall (test 2, Right). Saline (n = 9) and anisomycin (n = 8) groups. (N) Cell counting 8 d posttraining. c-Fos+/mCherry+ overlap (n = 3 per group). Chance at 0.77 (CA3) and 0.43 (BLA). (O) Optogenetic recall (Engagement activation light-on epochs, Left) plotted for saline and anisomycin groups. Overlap retention (connectivity, Right) plotted as a ratio of ChR2-induced engram reactivation relative to natural recall-induced engram reactivation. Data shown for 2, 5, or 8 d posttraining. Data are presented as mean ± SEM. Unless specified, statistical comparisons are performed using unpaired t tests; *P < 0.05, **P < 0.01, ***P < 0.001.
**Fig. 2.** Strong, but not weak, optogenetic stimulation of DG engram cells restores fear memory in retrograde amnesia. (A) Behavioral schedule. c-fos-tTA mice with AAV9-TRE-ChR2-eYFP injected into the DG and AAV9-TRE-mCherry injected into both CA3 and BLA were used. Mice were perfused 1 h postengram activation for cell-counting overlap. (B) Natural recall (test, n = 9 per group). (C) Ex vivo electrophysiological recordings of CA1 engram cells expressing AAV9-TRE-ChR2-mCherry (n = 6), using 25% (1.5–2.5 mW), 50% (4–6 mW), and 75% (7–9 mW) laser power (Left). Representative traces (Middle). Light-induced activation plotted as photocurrent (pA, Right). (D) Optogenetic recall using 25% laser. Significant freezing was observed in the saline group (n = 9), but not in the anisomycin group (n = 8). (E) Cell counting at 25% laser. c-Fos/ChR2 overlap in DG and c-Fos/mCherry overlap in CA3 and BLA (n = 5 per group). Chance at 0.38 (DG), 0.89 (CA3), and 0.62 (BLA). In DG, average ChR2 labeling was 6.3% (of DAPI) and cFos was 4.4%. Average mCherry labeling was 12.7% in CA3 and 7.9% in BLA. Average cFos was 9.33% in CA3 for both groups, and 5.8% for saline or 3.92% for anisomycin in BLA. (F) Optogenetic recall using 50% laser. Significant freezing was observed in saline (n = 10) and anisomycin groups (n = 10). (G) Cell counting at 50% laser. c-Fos/ChR2 overlap in DG, c-Fos/mCherry overlap in CA3 and BLA (n = 4 per group). Chance at 0.39 (DG), 0.82 (CA3), and 0.46 (BLA). (H) Optogenetic recall using 75% laser. Significant freezing was observed in saline (n = 7) and anisomycin groups (n = 8). (I) Cell counting at 75% laser. c-Fos/ChR2 overlap in DG, c-Fos/mCherry overlap in CA3, and BLA (n = 6 per group). Chance at 0.37 (DG), 0.91 (CA3), and 0.51 (BLA). (J) Optogenetic recall (engram activation light-on epochs, Left). Overlap retention (connectivity, Middle and Right) in CA3 and BLA, plotted as a ratio of ChR2-induced engram cell reactivation relative to natural recall-induced engram reactivation levels. Data shown for 25, 50, or 75% laser power. Data are presented as mean ± SEM. Statistical comparisons are performed using unpaired t tests; *P < 0.05, **P < 0.01, ***P < 0.001.
Fig. 3. Inactivation of engram cell connectivity downstream, but not upstream, prevents fear memory recall by optogenetic stimulation of DG engram cells in both control and retrograde amnesia. (A) Wild-type mice maintained on DOX were used. Virus mixture of AAV9-c-fos-tTA/AAV9-TRE-GFP-TetTox injected into the MEC to inactivate the upstream MEC-to-DG connection, and a mixture of AAV9-c-fos-tTA/AAV9-TRE-ChR2-mCherry into the DG, to label engram cells. (B and C) Sagittal sections of AAV9-TRE-GFP-TetTox labeling in MEC (B) and AAV9-TRE-ChR2-mCherry in DG (C). (D) GFP-TetTox counts from MEC and ChR2-mCherry counts from DG (n = 3 per group). (E) Behavioral schedule. In addition to TetTox mice, control mice injected with a virus mixture of AAV9-c-fos-tTA/AAV9-TRE-eYFP into MEC and a virus mixture of AAV9-c-fos-tTA/AAV9-TRE-ChR2-mCherry into the DG were also used. (F) Natural recall (test, Left, n = 10 per group). Saline-treated TetTox mice exhibited decreased freezing compared with saline eYFP. In a separate cohort, mice were perfused 1 h postnatural recall for overlap (Right). c-Fos\textsuperscript{+/mCherry\textsuperscript{+/+}} overlap in DG (n = 5 per group). Chance at 0.42. Average c-Fos labeling was 3.87% (of DAPI) in saline eYFP, and 2.9% in other groups. (G) Optogenetic recall (\textsim 10–12 mW at patch cords). Significant freezing was observed in all groups (n = 10 per group). (H) \textsuperscript{+/mCherry\textsuperscript{+/+}} overlap in CA3 (Left) and BLA (Right) of eYFP or TetTox mice following ChR2-induced recall (n = 4 per group). Chance at 0.95 (CA3) and 0.59 (BLA). Average mCherry labeling was 12.6% in CA3 and 8.2% in BLA. Average c-Fos was 10.05% in CA3 and 7.1% in BLA. (I) To inactivate downstream DG to CA3, a mixture of AAV9-c-fos-tTA/AAV9-TRE-GFP-TetTox/AAV9-TRE-ChR2-mCherry was injected into the DG. (J) Following the behavioral schedule in E, natural recall was performed (test, n = 13 per group). (K) Optogenetic recall (\textsim 10–12 mW at patch cords). Significant freezing was observed in both eYFP groups, but not in TetTox groups (n = 12 per group). (L) \textsuperscript{+/mCherry\textsuperscript{+/+}} overlap in CA3 (Left) and BLA (Right) of eYFP or TetTox mice following ChR2-induced recall (n = 5 per group). Chance at 0.91 (CA3) and 0.66 (BLA). Data are presented as mean ± SEM. Statistical comparisons are performed using unpaired t tests; \*P < 0.05, \**P < 0.01.
Fig. 4. Reversal of engram cell-specific spine deficits by PAK1 overexpression rescues natural memory recall in retrograde amnesia. (A and B) Dendritic spines from DG engram cells of saline (A) and anisomycin (B) groups. (C) Spine density post-CFC training from saline (n = 7,045 spines; n = 7 mice) and anisomycin (n = 5,789 spines; n = 6 mice). (D) Hippocampal CA1 sections from c-fos-tTA mice injected with AAV9-TRE-ChR2-eYFP. DAPI (Left) and ChR2-eYFP (Right). (E) ChR2-eYFP counts from CA1 sections (n = 4 per group). (F and G) Dendritic spines from CA1 engram cells of saline (F) and anisomycin (G) groups. (H) Spine density post-CFC training from saline (n = 5,122 spines; n = 6 mice) and anisomycin (n = 4,136 spines; n = 6 mice). (I) Engram cell-specific PAK1 overexpression. (J) Behavioral schedule. (K) Dendritic spines from anisomycin-treated CA1 engram cells of the PAK1-CA group (Left). Spine density 1 d or 5 d post-CFC training from eYFP (n = 3,895 spines; n = 5 mice) and PAK1-CA (n = 6,572 spines; n = 7 mice) groups (Right). Black dashed line indicates saline mice spine density (0.72). (L) Natural recall 1 d (test 1) and 5 d (test 2) posttraining (n = 15 per group). Anisomycin PAK1-CA mice displayed increased freezing during test 2. Black dashed line indicates saline mice freezing level (34%). Data are presented as mean ± SEM. Statistical comparisons are performed using unpaired t tests; *P < 0.05, **P < 0.01, ***P < 0.001.
overexpression of constitutive active PAK1 (PAK1-CA) in a memory engram cell-specific manner (Fig. 4I). In mice that received anisomycin infusions only into hippocampal CA1, 5 d after training, PAK1-CA overexpression restored CA1 engram cell spine density to control levels (Fig. 4J and K). Furthermore, this spine restoration in anisomycin-treated mice correlated with amelioration of long-term memory impairments observed during recall by natural cues (Fig. 4L, test 2), which was context-specific (Fig. 4L, neutral). These experiments identify a molecular genetic method to convert an engram from a silent state to an active state.

Discussion

An earlier study showed that, 1 d after training, synaptic strength and spine density in engram cells are augmented. However, an analysis of engram cells of mice that had received a blockade of posttraining protein synthesis indicated that memory retention did not depend on the maintenance of these changes in synaptic strength and spine density. Rather, the maintenance of learning-induced neuronal connectivity between engram cell ensembles correlated with memory retention (6). These findings support the idea that cellular consolidation is primarily for establishing memory retrievability rather than being an obligatory mechanism for storage of the representation per se (32, 33). In this study, we investigated this correlation more closely. First, we showed that the functional connectivity between engram cell ensembles lasted for a prolonged period after learning both in control mice and in anisomycin-treated mice. This was demonstrated in vivo by using the previously established overlap method of labeling upstream engram cells and downstream engram cells reactivated by activation of the former. Following the earlier demonstration of a significant overlap at 1 d after training (6), we have now shown that a similar degree of overlap is maintained at 5 and 8 d after training between DG and CA3, as well as DG and BLA, engram cells. Furthermore, we confirmed that this long-term maintenance correlates with that of optogenetic memory recall. These results indicate that, once a specific pattern of connectivity between engram cell ensembles is established during learning, it is stable for at least a week, even in the absence of posttraining protein synthesis. Second, in this study we demonstrated that for optogenetic memory recall to take place by activation of an engram cell ensemble (DGs), the connectivity between DGs, or in either normal or anisomycin-treated mice, intact connectivity with a downstream engram cell ensemble (CA3) is crucial, whereas connectivity with an upstream engram cell ensemble (MEC) is not. These results provide causal evidence for the notion that engram cell ensemble connectivity is the basis for retained memory.

In anisomycin-treated animals, engram cells are in a silent state: the state in which memory information is retained and retrievable by optogenetic stimulation, but not by natural recall cues. The crucial feature of silent engram cells is the lack of augmented synaptic strength and spine density due to a posttraining blockade of protein synthesis. The efficiency of natural recall would be greatly influenced by these synaptic features, and hence it can be attained as long as a certain threshold of functional connectivity between engram cell ensembles is reached. However, since the engram cell connectivity would also depend on the synaptic strength and abundance, one would predict that functional engram cell connectivity would be weakened in anisomycin-treated animals, and hence optogenetic recall from these mice would depend on the strength of blue light. Our data in Fig. 2 confirmed this prediction: when the strength of blue light was reduced, both memory recall and functional engram cell connectivity were reduced. Furthermore, the crucial link between spine density, engram cell connectivity, and natural recall was demonstrated by restoration of full connectivity and natural recall by PAK1 overexpression in animals that received anisomycin infusions into hippocampal CA1.

Silent engrams are not a phenomenon specifically associated with retrograde amnesia. In a mouse model of early Alzheimer’s disease, the hippocampal engrams are in a silent state that can be converted to an active state by repeated high-frequency stimulation of the engram cell synapses (22). In hippocampus-dependent episodic memory, engrams are formed rapidly on the day of learning not only in the hippocampus, but also in the prefrontal cortex (PFC). However, the PFC engrams are in a silent state and need to mature to acquire an active state, which takes many days and help from hippocampal engram cells. Conversely, the hippocampal engrams become silent as days go by (29). Furthermore, the longevity of social memory formed in ventral CA1 is relatively short-lasting—just several hours—but it continues to exist in a silent state for at least a few days (18). Thus, silent engrams are more routine than exceptional. Further studies on the properties of silent and active engrams and their mutual conversion would advance our understanding of the regulation of formation and retrieval of memory.

Materials and Methods

Animals. The c-fos-tTA transgenic mice were generated as described (4). The C57BL/6j wild-type mice were obtained from Jackson Laboratory. Mice and their controls were fed ad libitum and were socially housed in numbers of two to five littersmates until surgery. Following surgery, mice were singly housed. For behavioral experiments, all mice used for the experiments were male and 8–10 wk old at the time of surgery, had been raised on food containing 40 mg kg−1 doxycycline (DOX) for at least 1 wk before surgery, and remained on DOX food for the remainder of the experiments except for the target engram labeling days. An identical procedure was used for virus-mediated (22) engram labeling experiments (Fig. 3) in which wild-type male mice were raised on DOX. All experiments were conducted in accordance with the National Institutes of Health guidelines and the Massachusetts Institute of Technology Department of Comparative Medicine and Committee on Animal Care.

Viral Constructs. The pAAV-TRE-GFP-TetTox and pAAV-TRE-PAK1-CA plasmids were constructed by cloning the GFP-TetTox and PAK1-CA (Addgene #12212) fragments into an AAV backbone containing the TRE promoter sequence (4). The pAAV-c-fos-tTA construct generation was done as described previously (22). The pAAV-TRE-ChR2-eYFP, pAAV-TRE-eYFP, pAAV-TRE-Chr2-mCherry, and pAAV-TRE-mCherry constructs were previously described (4, 6). AAV vectors were serotyped with AAVV 1 coat proteins and packaged at the University of Massachusetts Medical School Gene Therapy Center and Vector Core or at the Massachusetts General Hospital. Viral titers were estimated at 5 × 1013 gc mL−1 for AAVV-c-fos-tTA, AAVV-TRE-ChR2-eYFP, and AAVV-TRE-eYFP, 1.2 × 1013 gc mL−1 for AAVV-TRE-Chr2-mCherry, 2 × 1013 gc mL−1 for AAVV-TRE-mCherry, and 3 × 1013 gc mL−1 for AAVV-TRE-GFP-TetTox and AAVV-TRE-PAK1-CA.

Surgery and Optic Fiber Implants. Mice were anesthetized with isoflurane or 500 mg kg−1 ketamine for stereotaxic injections, as described (22). Injections were targeted bilaterally to the DG (±2.0 mm anterior posterior [AP], ±1.3 mm medial lateral [ML], −1.9 mm dorsal ventral [DV]), MEC (−4.85 mm AP, ±3.36 mm ML, −2.75 mm DV), CA3 (−2.0 mm AP, ±2.2 mm ML, −2.2 mm DV), CA1 (−2.1 mm AP, ±1.5 mm ML, −1.4 mm DV), and BLA (−1.94 mm AP, ±3.1 mm ML, −4.65 mm DV). Injection volumes were 200 nl for DG and CA3, 300 nl for MEC and BLA, and 400 nl for CA1. A custom DG implant containing two optic fibers (200-mm core diameter; Dorian Lenses) was used. As criteria, we included only mice with virus expression limited to the target regions.

Immunohistochemistry. Mice were perfused transcardially with 4% paraformaldehyde. Fifty-micrometer coronal slices were prepared using a vibratome. Immunostaining was performed as described (22). Antibodies used for staining were as follows: to stain for ChR2-eYFP, GFP-TetTox, or eYFP alone, slices were incubated with primary chicken anti-GFP (1:1,000; Life Technologies) and visualized using anti-chicken Alexa-488 (1:500). For ChR2-mCherry, or mCherry alone, slices were stained using primary rabbit anti-RFP (1:1,000; Rockland) and secondary anti-rabbit Alexa-555 (1:500). c-Fos was stained with rabbit anti-c-Fos (1:500; Calbiochem) and anti-rabbit Alexa-488 (1:300).
To characterize the expression pattern of ChR2-eYFP, GFP-TetTox, eYFP, mCherry, c-Fos, and ChR2-mCherry in saline and anisomycin, or an equivalent volume of saline, was delivered intraperitoneally immediately after CFC training. For CA1-specific experiments, anisomycin was delivered via cannulae (400 nL of a 150 μg/mL stock). The engraving activation context had perspex floors, opaque triangular ceilings, red lighting, and 1% acetic acid scent. To disrupt cellular consolidation, 150 mg anisomycin/kg was administered 15 min after control meal termination. This work was supported by the RIKEN Brain Science Institute, the Howard Hughes Medical Institute, and the JPB Foundation (S.T.).

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