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Dynamics of Retrieval Strategies for Remote Memories

Inbal Goshen,[1] Matthew Brodsky,[1] Rohit Prakash,[1] Jenelle Wallace,[1] Viviana Gradinaru,[1] Charu Ramakrishnan,[1] and Karl Deisseroth[1,*]
[1]Department of Bioengineering, Department of Psychiatry and Behavioral Sciences, Howard Hughes Medical Institute, CNC Program, Stanford University, Stanford, CA 94305, USA
*Correspondence: deissero@stanford.edu
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SUMMARY

Prevailing theory suggests that long-term memories are encoded via a two-phase process requiring early involvement of the hippocampus followed by the neocortex. Contextual fear memories in rodents rely on the hippocampus immediately following training but are unaffected by hippocampal lesions or pharmacological inhibition weeks later. With fast optogenetic methods, we examine the real-time contribution of hippocampal CA1 excitatory neurons to remote memory and find that contextual fear memory recall, even weeks after training, can be reversibly abolished by temporally precise optogenetic inhibition of CA1. When this inhibition is extended to match the typical time course of pharmacological inhibition, remote hippocampus dependence converts to hippocampus independence, suggesting that long-term memory retrieval normally depends on the hippocampus but can adaptively shift to alternate structures. Further revealing the plasticity of mechanisms required for memory recall, we confirm the remote-timescale importance of the anterior cingulate cortex (ACC) and implicate CA1 in ACC recruitment for remote recall.

INTRODUCTION

The consolidation of remote memories relies on both synaptic processes on the timescale of minutes to hours, and circuit consolidation over weeks to years (Frankland and Bontempi, 2005; Squire and Bayley, 2007). Pioneering work on the circuitry of memory has shown that the process of long-term contextual fear memory consolidation requires early involvement of the hippocampus, followed by the neocortex. Specifically, these studies showed that hippocampal lesions impair recent memory 1 day after training, but the same lesions have no effect on remote memory several weeks after training (Anagnostaras et al., 1999; Bontempi et al., 1999; Debiec et al., 2002; Frankland et al., 2004; Kim and Fanselow, 1992; Kitamura et al., 2009; Maren et al., 1997; Maviel et al., 2004; Shimizu et al., 2000; Wang et al., 2003; Winocur et al., 2009). Such graded retrograde amnesia is also observed in human patients with medial temporal lobe injuries (Squire and Alvarez, 1995; Squire and Bayley, 2007). However, complete, nongraded amnesia has been reported in animals performing spatial memory tasks (Broadbent et al., 2006; Riedel et al., 1999) and in some human subjects. Indeed, studies causing extensive hippocampal damage have reported nongraded retrograde amnesia for fear conditioning (FC) (Sutherland et al., 2008; Wang et al., 2009; Winocur et al., 2007). This and other work has led to the "multiple trace theory" that the hippocampal memory trace is not replaced by the cortical one, but rather both memories are in continuous interplay, and the effect of hippocampal lesions may depend on both the nature of the task and the nature of the lesion (Cipolotti and Bird, 2006; Moscovitch et al., 2006; Winocur et al., 2010).

This pioneering work on the circuitry of memory has involved physical, pharmacological, and genetic lesion studies, which have greatly enhanced our understanding of neural systems. These methods are highly effective but typically involve tradeoffs between cellular and temporal precision. Elegant genetic interventions can be cell type specific (McHugh et al., 2007; Nakashiba et al., 2008) but are slow on the timescale of days. Pharmacological lesions enable higher temporal resolution on the timescale of minutes (Kitamura et al., 2009; Wiltgen et al., 2010) but are still slower than neurons and not typically cell specific. Optogenetics with microbial opsin genes (Boyden et al., 2005; Deisseroth et al., 2006; Zhang et al., 2007) enables both cell-type precision and temporal control on the millisecond timescale. To begin optogenetic exploration of the cellular and circuit underpinnings of long-term memory, we expressed the fast optogenetic inhibitor eNpHR3.1 (Gradinaru et al., 2010) bilaterally in excitatory neurons within the CA1 subfield of the dorsal hippocampus. As it is known that contextual FC but not auditory-cued FC relies on the hippocampus, whereas the amygdala is required for both contextual and auditory-cued FC (LeDoux, 2000; Maren, 2001; Maren and Quirk, 2004), we employed fiberoptic-mediated light delivery (Adamantidis et al., 2007; Aravanis et al., 2007) to these and other neural circuits of freely moving animals in the setting of contextual FC and recall, capitalizing on the circuit specificity of this behavior and well-defined temporal separation between different stages of memory (acquisition, consolidation, and recall).

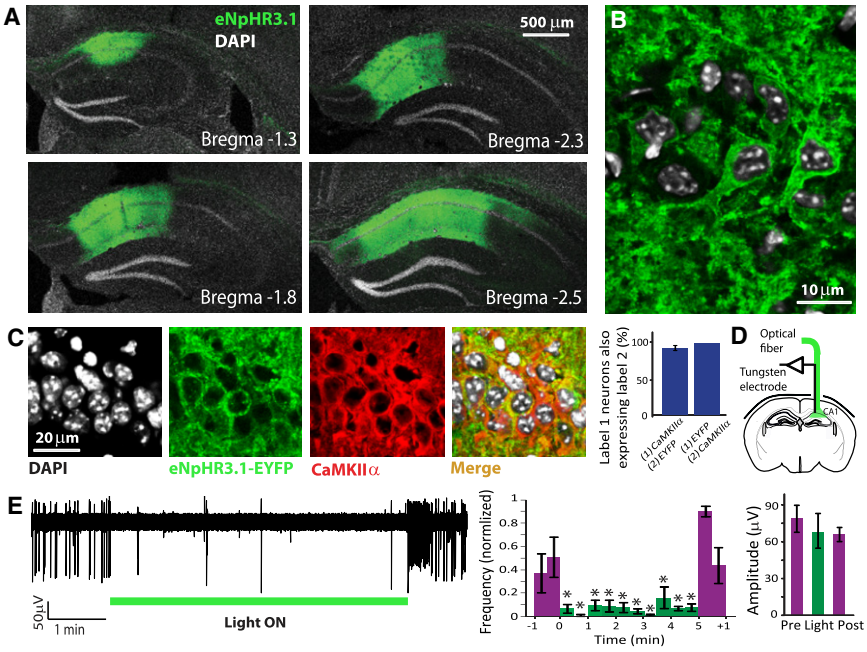

Figure 1. Specific Optogenetic Inhibition of Excitatory Neurons in Dorsal CA1 Reduces Neuronal Activity

(A) Double lentiviral injection resulted in eNpHR3.1 expression in CA1 only.

(B) eNpHR3.1 is expressed in the neuronal membrane around the soma, as well as in the apical and basal dendrites of CA1 neurons.

(C) CaMKIIα::eNpHR3.1 was expressed in 94% (458/486 cells, from three mice) of CA1 pyramidal neurons, with 100% specificity (all eNpHR3.1-EYFP cells were CaMKIIα positive).

(D) In vivo CA1 "optrode" recording setup.

(E) Illumination of CA1 neurons in eNpHR3.1-expressing mice resulted in a reversible reduction in spiking frequency (2.41 ± 1.1 Hz, 0.54 ± 0.4 Hz, and 4.23 ± 1.4 Hz; before, during, and after light administration, respectively, in five traces from two mice; $p < 0.001$), without affecting average spike amplitude (79.3 ± 11.11 μV, 68.80 ± 14.4 μV, and 66.82 ± 5.8 μV; before, during, and after light). A representative optrode recording trace as well as average frequency and amplitude are shown (mean ± standard error of the mean [SEM]).

For additional information about eNpHR 3.1 in the hippocampus, see Figure S1.

RESULTS

Specific Optogenetic Inhibition of Excitatory Neurons in Dorsal CA1 Reduces Neuronal Activity

We employed a lentiviral vector encoding eNpHR3.1 fused in-frame to enhanced yellow fluorescent protein (eNpHR3.1-EYFP) under control of the calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) promoter, selective for excitatory glutamatergic neurons. eNpHR3.1 is a truncated version of eNpHR3.0 that has a deletion of the intrinsic N-terminal signal peptide and that is similar to eNpHR3.0 in both the photocurrent and the hyperpolarization induced in neurons (Figures S1A and S1B available online). Stereotactic delivery of the CaMKIIα::eNpHR3.1 vector was found to result in CA1-specific expression (Figures 1A, 1B, and S1C). Within the transfected area, 94% of the CaMKIIα cells expressed eNpHR3.1, and the promoter provided essentially complete specificity as well (Figure 1C). To verify the physiological effect of eNpHR3.1 on CA1 neuronal activity, we performed optrode recordings in anesthetized mice (Figure 1D) and confirmed that continuous 561 nm illumination of excitatory CA1 neurons inhibited spiking in vivo in a temporally precise, stable, and reversible manner (Figure 1E).

CA1 Optogenetic Inhibition Blocks Contextual Fear Acquisition and Retrieval

Understanding of the involvement of the hippocampus in contextual FC is based on physical, pharmacological, and genetic lesions to this structure in which the interval between lesion and testing ranges from tens of minutes to several weeks. To test whether real-time optogenetic inhibition of CA1 could modulate memory formation, we delivered bilateral continuous green (561 nm) light to dorsal CA1 (Figure 2A) during FC training in all animals, thereby delivering CA1 inhibition in eNpHR3.1 but not control mice. Fear memory was assessed the next day in the absence of light, and the dorsal CA1 optogenetic inhibition during training was found to prevent contextual fear memory (Figure 2B, left). To test whether this effect was reversible, all mice were retrained in the same context without light and tested again on the next day; eNpHR3.1 mice exhibited intact contextual memory when no light was administered during training (Figure 2B, middle).

We next retested the same mice with light delivery during recall to examine whether dorsal CA1 optogenetic inhibition could also interfere with recall, and we found that the memory that had been present only the day before became unavailable for recall under illumination (Figure 2B, right). These experiments suggest a real-time involvement of CA1 excitatory cells in acquisition and recall of recent contextual fear memory. Importantly, eNpHR3.1 mice demonstrated intact auditory-cued fear memory acquisition following CA1 light inhibition during training (Figure 2C, left) and intact cued fear recall with illumination during the test (Figure 2C, right), demonstrating the functional specificity of the optogenetic manipulation in affecting only the hippocampus-dependent task.

Because exploration is critical for contextual fear acquisition (Fanselow, 1990; McHugh and Tonegawa, 2007), we measured exploration in the conditioning chamber during training with light and found no difference between eNpHR3.1 and control mice (Figure 2D). To verify that CA1 optogenetic inhibition had no anxiolytic effect, mice were tested for open-field exploration during light administration. No differences in path length (Figure 2E), velocity (Figure 2F), or the percent of time spent in the center of the field (a sign of anxiety-related behavior) were found between eNpHR3.1 and control mice (Figure 2G).

Finally, we bilaterally injected mice in the basolateral amygdala (BLA; Figure 2H) and found that we could optogenetically inhibit

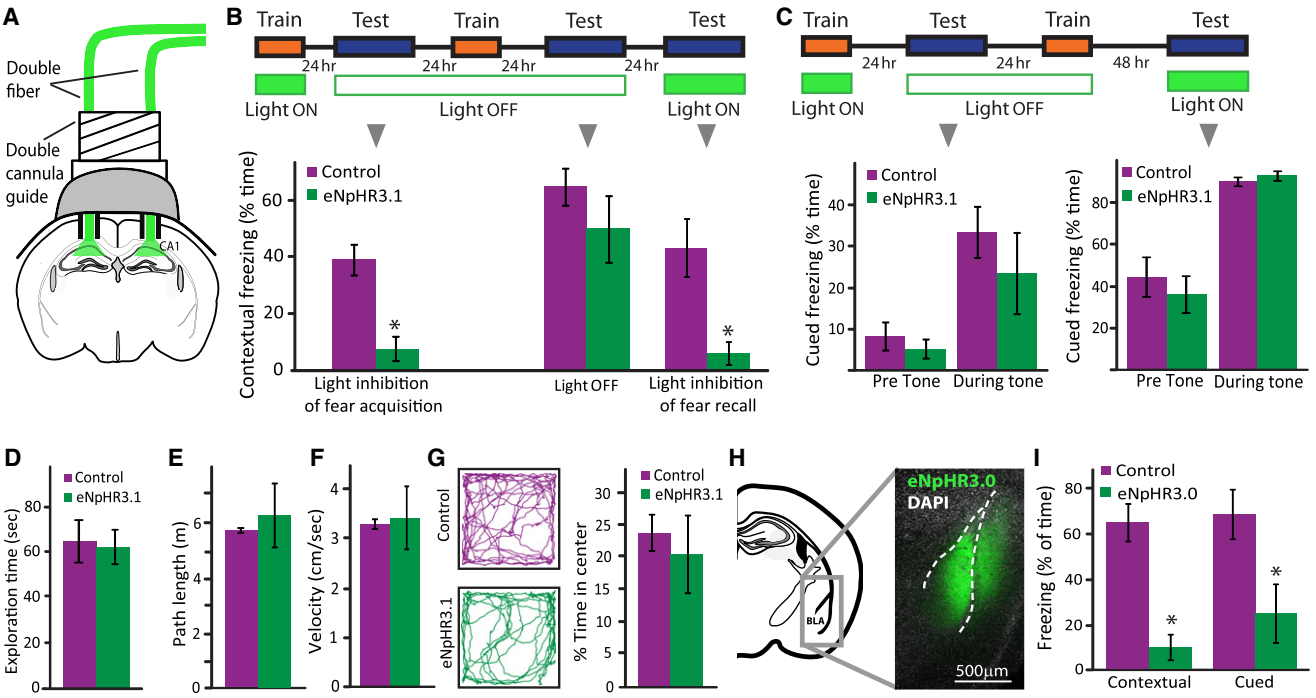

Figure 2. Real-Time CA1 Optogenetic Inhibition Blocks Contextual Fear Acquisition and Retrieval

(A) Bilateral in vivo light administration to CA1.

(B) Top: Training and illumination protocol. Bottom: CA1 optogenetic inhibition during training ("Light ON") prevented fear acquisition in eNpHR3.1 mice (n = 5) compared to controls (n = 4) (39% ± 5.4% versus 7.6% ± 4.3% freezing; $p < 0.005$). When retrained without illumination ("Light OFF"), the same mice demonstrated intact contextual memory (64.6% ± 6.6% versus 49.7% ± 11.7% freezing; $p > 0.5$). This fear memory became unavailable for recall upon light administration during testing in eNpHR3.1 mice (42.6% ± 10.1% versus 5.94% ± 4.1% freezing; $p < 0.01$).

(C) CA1 optogenetic inhibition had no effect on either acquisition (left) or recall (right) of auditory-cued fear memory in eNpHR3.1 mice (n = 5) compared to controls (n = 4).

(D) Optogenetic inhibition had no effect on exploration of the context before conditioning in eNpHR3.1 mice (n = 5) compared to controls (n = 4).

(E–G) In a novel environment, control (n = 6) and eNpHR3.1 (n = 4) mice explored the field with (E) similar path lengths (564 ± 9 and 618 ± 114 cm) and (F) similar speeds (3.3 ± 0.1 versus 3.43 ± 0.6 cm/s). There was no effect on anxiety (G), as the percent of time that control and eNpHR3.1 mice spent in the center of the open field was similar (23.8% ± 2.76% versus 20.46% ± 5.97%; $p > 0.5$). Representative exploration traces are presented.

(H) eNpHR3.0 expression in the BLA.

(I) Light administration to the BLA resulted in impaired contextual (65.5% ± 7.2% versus 9.6% ± 5.5% freezing; $p < 0.001$) and auditory-cued (69.5% ± 9.6% versus 24.5% ± 13% freezing; $p < 0.05$) memory acquisition in eNpHR3.0 (n = 4) mice, compared to controls (n = 9).

Data presented as mean ± SEM.

both contextual and auditory-cued FC acquisition (Figure 2I), as expected from prior findings that acquisition and expression of fear depend on the amygdala (LeDoux, 2000; Maren and Quirk, 2004). Together this constellation of findings supports the validity of the optogenetic system and a wide array of major prior findings in the memory literature by directly demonstrating the real-time role of the hippocampus in acquisition and recall.

CA1 Optogenetic Inhibition Reversibly Interferes with Remote Fear Memory Recall

We next explored the role of the hippocampus in remote recall. We trained a group of mice and tested recall 4 weeks later (Figure 3A), far into the remote phase when no hippocampus involvement is expected. Surprisingly, we found that CA1 inhibition during recall blocked remote fear memory. This interference was reversible; when the same mice were retested on the next day without illumination, fear memory was fully expressed (Figure 3A). Moreover, eNpHR3.1 mice demonstrated intact remote auditory-cued fear recall with illumination during the cued test (Figure 3B), demonstrating that fear expression mechanisms remained intact. To test whether the hippocampus would be involved in contextual fear recall as long as a memory trace could be practically detected in our hands, we trained two additional populations of mice and tested recall 9 or 12 weeks later. We found that CA1 inhibition during recall blocked remote fear memory even after these very long intervals (Figures 3C and 3D).

These results point to ongoing involvement of the hippocampus in remote contextual fear memories, suggesting that the intact hippocampus can still act as the default activator of the memory trace. However, we were able to obtain pharmacological data consistent with prior physical, pharmacological, or genetic lesions to the hippocampus, in which the interval between lesion and recall test ranges from tens of minutes to several weeks (Anagnostaras et al., 1999; Kim and Fanselow,

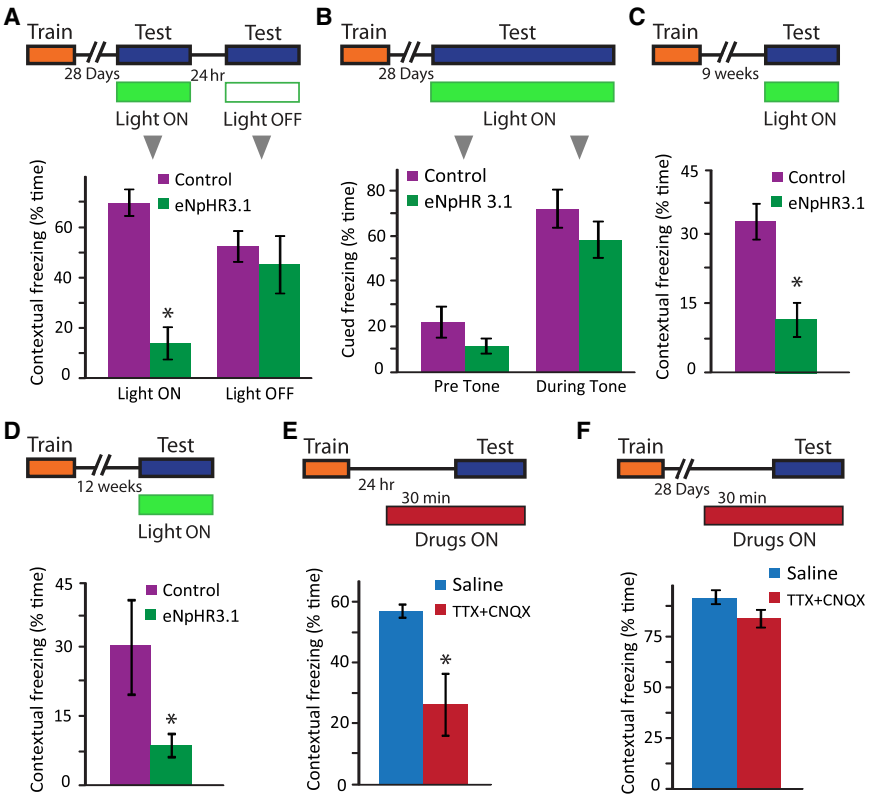

Figure 3. CA1 Optogenetic Inhibition Reversibly Interferes with Remote Fear Memory Recall

(A) CA1 optogenetic inhibition prevented remote memory ($p < 0.0001$; control, n = 14, 69.8% ± 5.3% freezing; eNpHR3.1, n = 6, 14% ± 6.4% freezing). This disruption was reversible, as when the same mice were reintroduced to the conditioning context with no illumination, they demonstrated intact fear responses (52.45% ± 6.0% versus 45.18% ± 11.5% freezing; $p > 0.5$).

(B) Remote auditory-cued fear was not affected (72.4% ± 8.4 versus 58.77% ± 7.9% freezing to the tone; $p > 0.5$).

(C and D) CA1 optogenetic inhibition impaired recall of ultra-remote memory that was acquired (C) 9 weeks earlier ($p < 0.005$; control, n = 9, 31.8% ± 3.8% freezing; eNpHR3.1, n = 6, 11.3% ± 3.6% freezing) or (D) 12 weeks earlier ($p < 0.005$; control, n = 6, 30.3% ± 10.3% freezing; eNpHR3.1, n = 8, 8.6% ± 2.6% freezing).

(E) Pharmacological hippocampal inhibition by TTX and CNQX 1 day after conditioning prevented recent fear recall (saline, n = 5, 56.86% ± 1.9% freezing; TTX+CNQX, n = 4, 26.05% ± 10.23% freezing; $p < 0.05$).

(F) TTX and CNQX administration 1 month after conditioning did not affect remote fear recall (saline, n = 8, 93.93% ± 2.54% freezing; TTX+CNQX, n = 9, 83.8% ± 4.4% freezing; $p > 0.05$).

1992; Shimizu et al., 2000; Wiltgen et al., 2010). Indeed, in our own hands as well, pharmacological inhibition of the hippocampus using TTX and CNQX, as previously reported (Kitamura et al., 2009), disturbed only recent (Figure 3E) but not remote (Figure 3F) fear recall, with one possible interpretation being that the speed of optogenetic inhibition here permits necessity testing without allowing expression of compensatory mechanisms.

Precise but Not Prolonged CA1 Optogenetic Inhibition Blocks Remote Contextual Fear Recall

To test this hypothesis that temporal precision is a critical factor accounting for the discrepancy between optogenetic and pharmacological findings, we repeated the remote optogenetic experiment either with illumination limited to the duration of the test as before (Figure 4A, "precise") or with prolonged illumination for 30 min before testing and during the test to mimic a slower intervention and allow time for putative compensatory mechanisms to be engaged (Figure 4A, "prolonged"). Precise optogenetic inhibition impaired remote memory, whereas prolonged inhibition had no detectable effect (Figure 4A). Furthermore, when mice from the prolonged group were retested on the next day with precise light administration, inhibited recall was observed (Figure 4A, right).

To validate these results, we trained mice and tested 1 day later with prolonged illumination, which confirmed that prolonged optogenetic CA1 inhibition, which had no effect on remote memory, still could block recent memory. We found that prolonged optogenetic inhibition was a fully potent intervention, significantly inhibiting recent fear memory recall (Figure 4B) just as with the pharmacological effect (Figure 3D). Additionally, we performed whole-cell patch clamp recordings (in slices prepared from the prolonged group in Figure 4A), which revealed that the ability of eNpHR3.1 to suppress spiking was stable throughout 30 min recording periods, as expected (Gradinaru et al., 2010), and was fully reversible (Figure 4C). Although it cannot be ruled out that in vivo inhibition may be less efficient than in this slice preparation, in vivo prolonged inhibition efficacy in the awake, freely moving state is supported by both inhibition of recent recall (Figure 4B) and *c-fos* measures of activity, as described in Figure 6 below.

We next sought to determine whether the remote fear memory expression could be interrupted even in the midst of a behavioral session. We trained another population of mice and characterized the cohorts with memory testing 5 weeks after contextual FC, first verifying persistence of the memory trace (without light during testing, observing similar performance in both eNpHR3.1 and control groups as expected; Figure 4D, left). On the next day, the same mice were tested under illumination, and the eNpHR3.1 group failed to recall the memory (Figure 4D, left). This effect in turn was fully reversible, as on the next day, when tested without light delivery, eNpHR3.1 mice demonstrated intact contextual memory (Figure 4D, right); however, as soon as the light was delivered again within this session, after the mice had already recalled the aversive context and expressed fear, the fear response immediately ceased (Figure 4D, right; Movies S1 and S2) in eNpHR3.1 but not control animals.

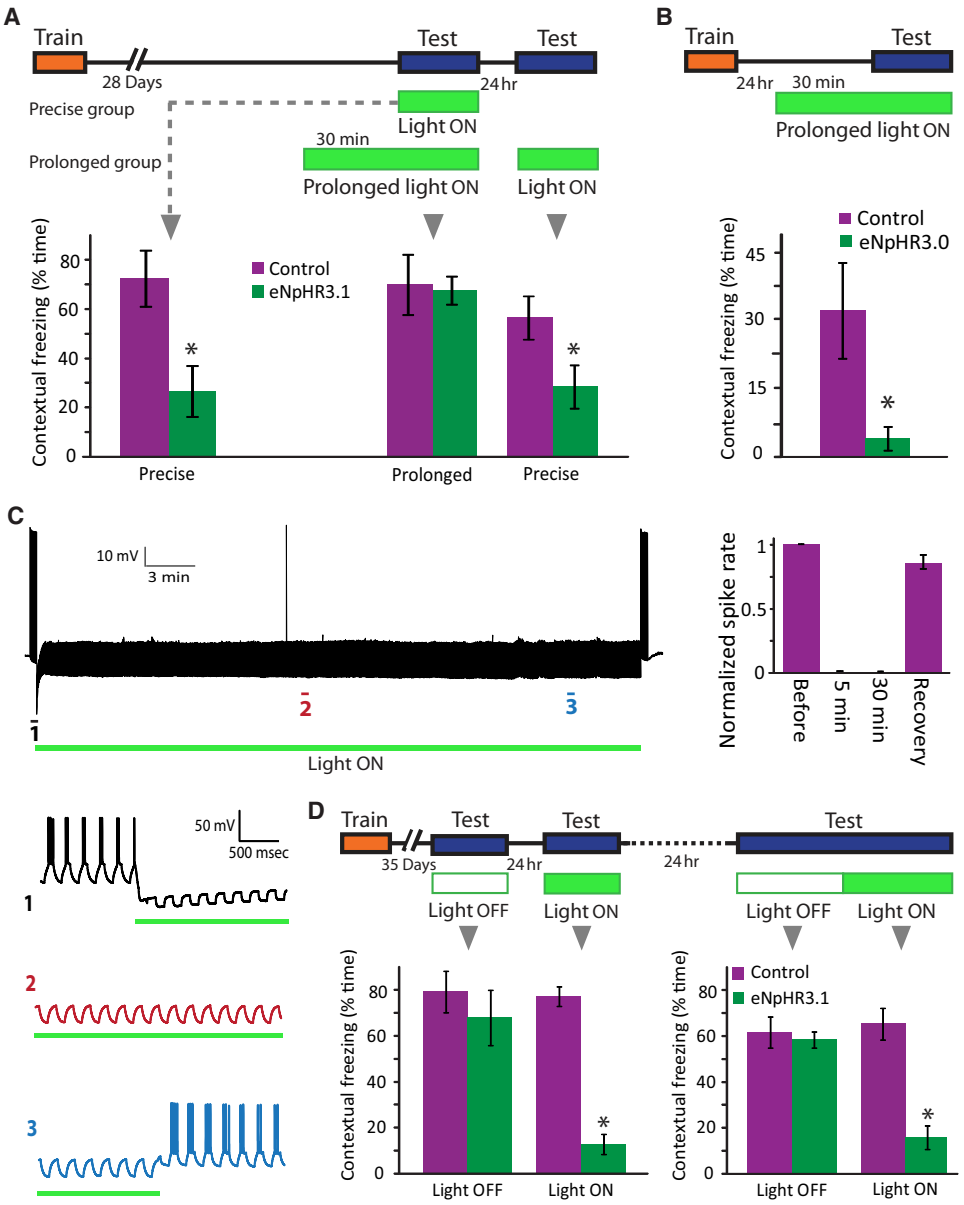

Figure 4. Precise but Not Prolonged CA1 Optogenetic Inhibition Blocks Remote Contextual Fear Recall

(A) CA1 optogenetic inhibition prevented remote fear recall only when light was administered precisely during testing (precise group, left: control, n = 4, 72.65% ± 11.5% freezing; eNpHR3.1, n = 8, 26.9% ± 10.4% freezing; $p < 0.01$), but not when the light was ON continuously for 30 min before (as well as during) the test (prolonged group, middle: control, n = 3, 70.13% ± 12.2% freezing; eNpHR3.1, n = 4, 67.7% ± 5.6% freezing; $p > 0.05$). When the prolonged group mice were retested the next day with precise light, their recall was disrupted (prolonged group, right: 55.5% ± 8.5 versus 27.6% ± 8.6% freezing; $p < 0.05$).

(B) Prolonged light prevented recall of recent memory (control, n = 7, 32.2% ± 10.6% freezing; eNpHR3.1, n = 3, 4% ± 2.6% freezing; $p < 0.05$).

(C) eNpHR3.1 continuously and potently prevented evoked spiking for 30 min in brain slices, as shown in the recording trace. Detailed traces of inhibition onset (1) mid-inhibition (2), and recovery (3) are presented on the bottom left. Averaged percent of successful evoked spiking is shown (n = 4 mice, 10 cells).

(D) Left: Remote fear memory that was efficiently recalled (control, n = 8, 79.0% ± 8.9% freezing; eNpHR3.1, n = 6, 67.8% ± 12.1% freezing; $p > 0.5$) was no longer available for recall under CA1 optogenetic inhibition (77.2% ± 4.3% versus 12.8% ± 4.4% freezing; $p < 0.0001$). Right: This disruption was fully reversible (61.5% ± 6.7% versus 58.3% ± 3.5% freezing; $p > 0.5$), and when illumination was introduced again in the middle of the testing trial, after the memory was already recalled, the fear response abruptly ceased (65.2% ± 6.9 versus 15.9% ± 5.2% freezing; $p < 0.001$).

To see representative movies of the mice analyzed in this experiment, please see Movies S1 and S2.

Together these data may unify certain disparate findings, by supporting prior work in suggesting that the remote memory trace is not stored only by these targeted hippocampal neurons (as when given enough time to compensate for hippocampal inactivation, the memory trace can still be retrieved, presumably by other structures in line with previous reports) but at the same

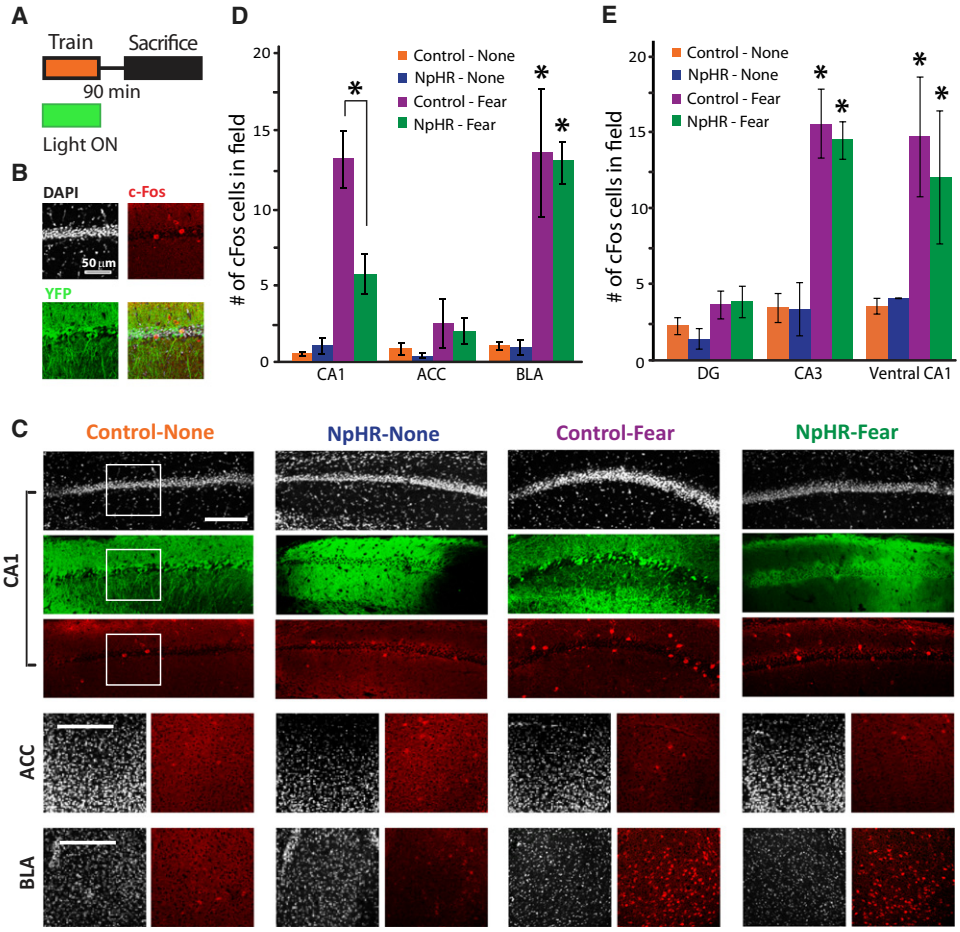

Figure 5. Brain-wide Mapping of Circuit Activity Controlled by the Hippocampus during Fear-Conditioning Acquisition

(A) Mice were fear-conditioned under light delivery, and brains were collected 90 min later.

(B) Slices were stained for c-Fos (red) and DAPI (n = 2 to 4 mice, 6 to 15 slices/group); expression of YFP control and eNpHR3.1 are shown in green.

(C) Representative images of CA1, ACC, and BLA are shown. White scale bars: 150 μm.

(D) CA1 optogenetic inhibition during FC reduced c-Fos expression in CA1 ($p < 0.05$) but not in ACC. In the BLA, activity levels were similarly elevated in control and eNpHR3.1 mice ($p < 0.001$).

(E) Optogenetic inhibition of dorsal CA1 did not affect activity in the DG, CA3, or ventral CA1. FC training resulted in a significant increase in c-Fos expression in CA3 and ventral CA1 of both control and eNpHR3.1 mice ($p < 0.01$ for all comparisons).

Data presented as mean ± SEM.

time revealing the surprising finding that the intact hippocampus may be a default activator of the remote memory trace and actively participates in its maintenance throughout the recall session.

Brain-wide Mapping of Circuit Activity Controlled by the Hippocampus during Remote Recall

Previous studies of the expression of immediate-early gene products (e.g., zif268 and c-Fos) have indicated that the transition from recent to remote memory can be accompanied by a decrease in recruited hippocampal activity and an increase in recruited neocortical activity in ACC and prefrontal cortex (Frankland et al., 2004; Hall et al., 2001; Maviel et al., 2004). To extend this activity mapping approach to the setting of CA1 optogenetic control, we inhibited CA1 during training or remote recall and assessed induction of c-Fos across the entire brain (Figures 5 and 6). With illumination during training, eNpHR3.1 mice demonstrated markedly reduced c-Fos expression specifically in CA1 compared with trained control animals (Figures 5A–5D) but showed BLA activity equivalent to that of trained controls (Figure 5D), revealing the expected hippocampus-independent engagement of fear circuitry during training. Noninfected hippocampal areas (dentate gyrus [DG], CA3, and ventral CA1) showed c-Fos levels comparable to those of controls following FC even in the setting of dorsal CA1 inhibition (Figure 5E). No significant changes in ACC activity levels were observed at this time point (Figure 5D), as previously reported (Bontempi et al., 1999; Frankland et al., 2004; Maviel et al., 2004).

Another group of mice was conditioned and re-exposed to the context 28 days later in the presence or absence of CA1 precise or prolonged optogenetic inhibition (Figure 6A); as before, the eNpHR3.1 mice exposed to precise light demonstrated impaired

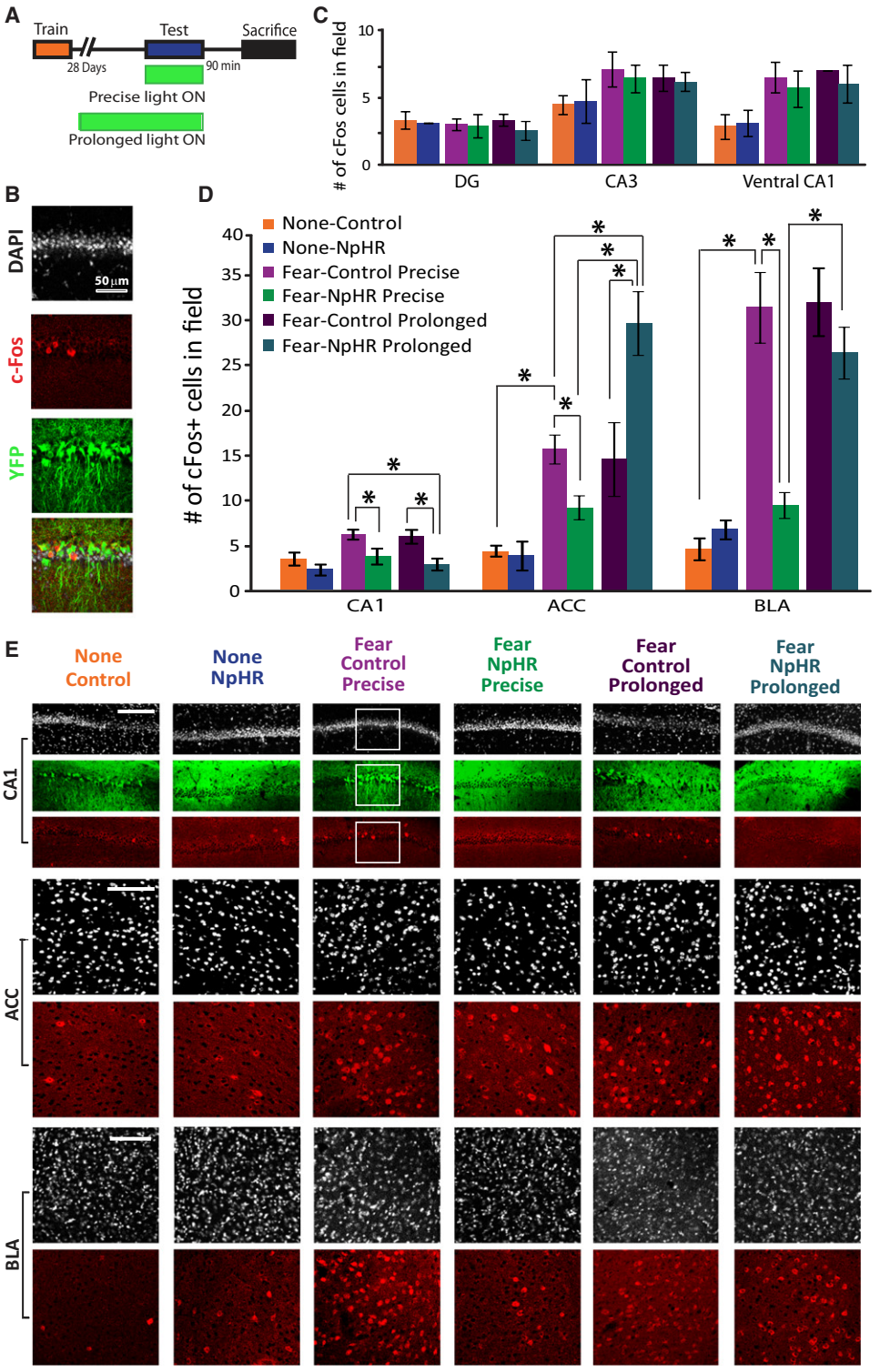

Figure 6. Brain-wide Mapping of Circuit Activity Controlled by the Hippocampus during Remote Recall

(A) Mice were conditioned and tested 28 days later, and brains were collected 90 min after testing.

(B) Slices were stained for c-Fos and DAPI (n = 3 to 6 mice, 6 to 25 slices/group). Expression of YFP control and eNpHR3.1 are shown in green.

(C) Remote recall caused no significant changes in c-Fos levels in DG, CA3, or ventral CA1, and no changes between eNpHR3.1 and control mice were observed in these regions.

(D) Remote recall led to increased activity levels in ACC ($p < 0.005$) and BLA ($p < 0.005$). Precise CA1 light inhibition during testing completely blocked CA1 activity ($p < 0.05$) and significantly reduced ACC and BLA activity ($p < 0.05$ for both) compared to control. Prolonged light inhibition blocked CA1 activity ($p < 0.05$) as

remote recall (Figure S2A). Ninety minutes later, the brains were collected and stained for c-Fos (Figure 6B) to capture putative memory-related brain-wide activity patterns under control of the hippocampus at this remote time point. Modest CA1 activity (Figures 6D and 6E) was observed, consistent with a highly efficient and sparse representation of the memory trace at the remote time point, but activity in these CA1 cells appeared to be involved in recruiting brain-wide activity, as the increase in ACC activity observed in control mice at this remote time point was reduced in eNpHR3.1 mice. Moreover, activated cell populations in the BLA were observed in control mice (which recognized the context and expressed fear; Figure S2A) but not in the eNpHR3.1 mice (which were unable to express the fear memory; Figures 6D, 6E, and S2A), suggesting involvement of CA1 in recruiting brain-wide fear memory activity patterns at this remote time point.

Additional observations point to the specificity of this CA1-recruited population at the remote time point. eNpHR3.1 mice showed normal activation in nontransduced hippocampal areas (DG, CA3, and ventral CA1; Figure 6C), no changes in parietal cortex activity (Figure S2B), and elevation in prefrontal cortex activity equivalent to that of controls (Figure S2B). A very different, novel pattern of brain activity emerged in eNpHR3.1 mice that were exposed to prolonged CA1 inhibition at the remote time point, a condition under which fear memory is not impaired (Figures 6B–6E and 4A). First, BLA activity was as high as the control activity level (Figures 6D and 6E), in accord with the robust display of fear and despite the reduced dorsal CA1 c-Fos activity (which was comparable to the low levels observed with precise light, further supporting the stable efficacy of the opsin over prolonged illumination in the behavioral setting). Finally and remarkably (Figures 6D and 6E), with prolonged CA1 inhibition, remote ACC activity not only was as potent as in the absence of inhibition but actually surpassed the levels seen in uninhibited controls, suggesting increased activity compensating for hippocampal inactivation. Together these data point to a surprising causal role for a restricted population of CA1 neurons in organizing the brain-wide activity patterns associated with remote contextual memory.

Optogenetic Inhibition of ACC Inhibits Remote but Not Recent Contextual Memory

Because the population of CA1 neurons active during remote memory appeared necessary for fully organizing ACC neuronal activity, and because previous research has implicated the ACC in remote fear memory storage (Bontempi et al., 1999; Frankland et al., 2004; Maviel et al., 2004), we next explored optogenetic inhibition of memories by targeting ACC directly (Figure 7A) either 1 day or 1 month following contextual FC. In accordance with previous studies (Frankland et al., 2004), optogenetic inhibition of ACC had no effect on recent memory but impaired remote memory (Figure 7B). To verify that optogenetic inhibition in ACC was indeed efficacious on the local circuit as well as the behavioral level, we examined c-Fos expression in ACC in the settings of recent and remote fear recall and found a significant increase in ACC neuronal activity in control mice, which was abolished in mice expressing eNpHR3.0 in ACC (Figure 7C). We then repeated the behavioral experiment in a new group of mice but this time delivered prolonged illumination. We found that this optogenetic inhibition of ACC impaired remote memory, again demonstrating efficacy of prolonged illumination for inhibition in the behavioral setting, but had no effect on recent memory (Figure 7D).

Finally, we targeted another major cortical input region, the olfactory bulbs (OB; Figure S3A), and tested the effect of OB optogenetic inhibition on recent and remote recall. We found no effect at either time point (Figure S3B). This result at once demonstrates that a sudden drop in a major source of synaptic input to cortex need not nonspecifically influence recall (without ruling out a contribution from such an effect) and also points to the specificity of ACC in remote memory (consistent with prior work). Together, these findings support the remote-memory importance of neocortex and also illustrate that even following cortical reorganization, there may exist a default requirement for the hippocampus in recalling remote memory traces.

DISCUSSION

Here we have leveraged the temporal resolution of optogenetics to probe the processes underlying recall of remote memories. Human studies on amnesia following medial temporal lobe damage have yielded mixed results—a temporal gradient for retrograde amnesia was reported in some studies, in which only recent memories were observed to be lost, but many other human studies report memory loss with no temporal gradient. Thus, in light of the complex animal and clinical literature, the possibility of a default role for the hippocampus in remote contextual memory recall is intriguing (Cipolotti and Bird, 2006; Moscovitch et al., 2006; Nadel and Moscovitch, 1997; Squire and Alvarez, 1995; Squire and Bayley, 2007; Winocur et al., 2010). Controlled animal models for retrograde amnesia have shown a time-limited role of the hippocampus in contextual fear memory (Anagnostaras et al., 1999; Bontempi et al., 1999; Debiec et al., 2002; Frankland et al., 2004; Kim and Fanselow, 1992; Kitamura et al., 2009; Maren et al., 1997; Maviel et al., 2004; Shimizu et al., 2000; Wang et al., 2009; Winocur et al., 2009), unless very extensive lesions were induced. In such cases, complete amnesia was observed, including both hippocampal-dependent contextual fear and hippocampal-independent auditory-cued fear (Sutherland et al., 2008). Surprisingly, we found that precise real-time inhibition of the CA1 region, sparing other hippocampal regions such as dorsal DG and CA3 and ventral CA1, is sufficient to impair remote recall. These data indicate that lesion magnitude may not be the only crucial parameter. Rather, temporal precision is also crucial; when time to compensate for the absence of hippocampal output is allowed, remote

potently as precise illumination, had no effect on BLA activity compared to control but significantly increased it compared to precise inhibition ($p < 0.05$), and significantly increased ACC activity compared to both precise inhibition ($p < 0.005$) and control ($p < 0.05$).
(E) Representative images of CA1, ACC, and BLA following remote recall are shown.
White scale bars: 150 μm. See also Figure S2.

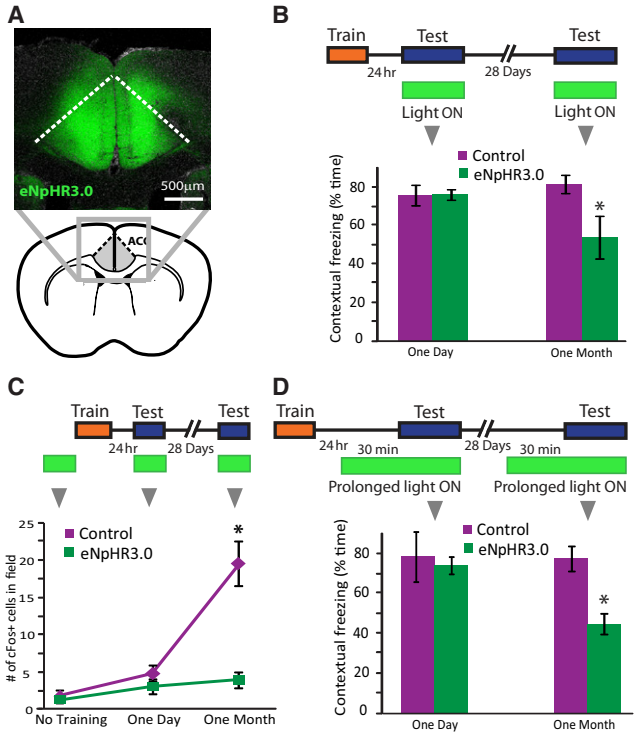

Figure 7. ACC Optogenetic Inhibition Disrupts Remote but Not Recent Fear Memory Recall

(A) eNpHR3.0 expression in the ACC.

(B) Precise light administration resulted in inhibition of remote (control, n = 5, 81.6% ± 4.9% freezing; eNpHR3.0, n = 5, 53.8% ± 11% freezing; $p < 0.05$) but not recent (75.9% ± 5.4% versus 76% ± 2.9% freezing) memory recall.

(C) Remote recall 28 days after conditioning resulted in a significant increase in ACC c-Fos expression in control mice compared to recent recall and no training ($p < 0.05$ for both; n = 2–3 mice, 8–18 slices/group). This increase was completely blocked by light inhibition of ACC in eNpHR3.0 mice ($p < 0.05$).

(D) Prolonged light in ACC also resulted in inhibition of remote (control, n = 3, 78.0% ± 6.2% freezing; eNpHR3.0, n = 8, 45.0% ± 5.2% freezing; $p < 0.05$) but not recent (78.5% ± 12.7% versus 74.3% ± 4.3% freezing) memory recall.

For control data, see Figure S3.

memory recall is no longer impaired. This is true for remote memory, after presumptive extrahippocampal consolidation, but not for recent memory, which may rely solely on hippocampal memory traces.

The idea that the hippocampus could be normally required for activation of neocortical memory traces is further supported by the brain-wide activity inferences with c-Fos. In agreement with previous studies, we observed an increase in ACC activity with remote recall. We also showed that CA1 inhibition during remote recall reduced this neuronal activity in ACC. However, when given enough time to compensate for the absence of hippocampal activity, ACC activity not only returned to but exceeded control levels, suggesting active compensation for hippocampal inactivation. Previous studies reported no detectable increase in hippocampal activity following remote fear recall (Bontempi et al., 1999; Frankland et al., 2004; Maviel et al., 2004); indeed we also found only modest effects upon exposure to the context, suggesting sparse and efficient storage of relevant information by the remote time point. A decrease in hippocampal activity in the transition from recent to remote memory was also previously observed for recall of a hippocampus-dependent spatial task (Maviel et al., 2004). Together these data may suggest that whereas increased hippocampal activity levels will be observed while memory storage processes are still taking place, hippocampal coding becomes more efficient in the remote phase, and basal or slightly increased activity levels are sufficient for the activation of a memory trace, especially after this trace is supported by additional structures such as the ACC.

The contribution of the ACC appears to be important enough that the hippocampus alone cannot independently support the full remote memory in contrast to the recent time point but relies on the ACC at least partially, as seen by the impaired remote memory in the presence of ACC inhibition. This finding in itself, however, does not prove or disprove the possibility that the hippocampus in the remote phase can function in part as an information conduit conveying some aspects of perception of the context to the ACC. Our finding that both CA1 and ACC inhibition interfere with remote recall could also support the "transformation" or "building on multiple trace theory," suggesting that in the process of system-wide consolidation, the memory is not merely copied from hippocampus to cortex but rather transformed, and that both memories remain available, with continuous interplay (Nadel and Moscovitch, 1997; Winocur et al., 2007, 2009, 2010). In this setting it is interesting to note that hippocampal lesions in animals induce retrograde failures in spatial memory (Broadbent et al., 2006; Martin et al., 2005), albeit in studies wherein the spatial-navigation role of the hippocampus was operative and required at the same time. We show here that even for a context recognition task, where no navigation is required, the hippocampus may still be the default activator of the memory trace, which may find antecedents in prior evidence that many place cells in CA1 remap in response to fear conditioning (Moser et al., 2008; Moita et al., 2004). As the task of contextual recall requires a comparison between the present perception of the context and stored representations, it may be suggested that real-time inhibition of CA1 interferes with the perception of the context during testing, rather than the recognition of the context from previous training, and that under prolonged illumination, perception rather than recall is recovered. Although we cannot rule out a theoretical recovery of perception, we would have expected such an effect to occur similarly in both remote and recent memory. We found, however, that only remote memory becomes available under prolonged inhibition, whereas both precise and prolonged CA1 inhibition similarly interfere with recent memory recall. These findings suggest that the recovered component is not perception, but we cannot entirely exclude this possibility because perception recovery could change following systems consolidation.

When remote memories are retrieved, the traces become available for reconsolidation, which may induce susceptibility to disruption but also may strengthen the trace (Dudai, 2006; Morris et al., 2006; Nader and Hardt, 2009; Tronson and Taylor, 2007; Wang and Morris, 2010). We find that optogenetic inhibition of a remote memory that was never recalled in the conditioning context is potent and completely reversible, suggesting that reconsolidation changes need not operate under these

conditions. On the other hand, remote optogenetic CA1 inhibition is potent enough to block the recall of a presumably reconsolidated memory trace that was recalled the previous day with no interference (Figure 4D). This reversible interruption of even a stable remote memory, in real-time and after retrieval/reconsolidation, supports an emerging view that pathology-inducing contextual memories or associations are susceptible to potential experimental or therapeutic interventions.

Finally, we note that among other implications, the results described here could be generally relevant to a class of often-hypothesized (but difficult to prove) interpretations of global activity measures (e.g., BOLD fMRI or c-Fos) in pathological states, as we have shown that indeed elevated correlates of activity in a particular brain region (in this case ACC) can actually represent not the pathological process itself but rather a recruited compensation to promote the underperforming process that the brain region normally supports. More broadly, these findings point to a potential remarkable dynamism in mammalian cognitive processes, in which underlying neural processes can adaptively shift the default circuits recruited. In summary, these findings and the high-speed methods presented here can serve as a basis for future studies examining the role of specific neuronal populations in cognitive and neuropsychiatric processes, enabling temporally, genetically, and spatially resolved dissection of the underlying neuronal circuits.

EXPERIMENTAL PROCEDURES

Subjects

C57BL6 mice aged 6 to 8 weeks (Charles River) were maintained on a reversed 12 hr light/dark cycle and given food and water ad libitum. Experimental protocols were approved by Stanford University IACUC and meet guidelines of the National Institutes of Health guide for the Care and Use of Laboratory Animals.

Virus Production

The CaMKIIα-eNpHR3.1-EYFP lentivirus for in vivo injection was produced as previously described (Gradinaru et al., 2010; Zhang et al., 2007). The adeno-associated virus (AAV) CaMKIIα-eNpHR3.0-EYFP plasmid was constructed by cloning eNpHR3.0-EYFP into an AAV backbone carrying the CaMKIIα promoter using BamHI and EcoRI restriction sites. The recombinant AAV vectors were serotyped with AAV5 coat proteins and packaged by the Vector Core at the University of North Carolina; titers were 2×10^{12} particles/ml. The maps for AAV CaMKIIα::eNpHR3.0 and Lenti CaMKIIα::eNpHR3.1 are available online at http://www.optogenetics.org.

Stereotactic Virus Injection, Cannula/Patchcord Implantation, and Light Delivery

Mice were anesthetized with isoflurane, and the head was placed in a stereotactic apparatus (Kopf Instruments, Tujunga, CA, USA). A small craniotomy was performed, and the virus was delivered using a 10 μl syringe and a thin 34 gauge metal needle (World Precision Instruments, Sarasota, FL, USA). The injection volume and flow rate (1 μl at 0.1 μl/min) were controlled by an injection pump (WPI). After injection the needle was left in place for 5 additional minutes and then slowly withdrawn. For CA1 optogenetic inhibition, concentrated lentivirus carrying CaMKIIα::eNpHR3.1-EYFP was microinjected into two sites in the CA1 (1 μl/site) of both left and right hippocampus (site one: anteroposterior [AP] −1.5 mm from bregma, mediolateral [ML] ±1 mm, dorsoventral [DV] −1.5 mm; site two: AP −2.5 mm, ML ±2 mm, DV −1.5 mm). A bilateral guide cannula (2.5 mm center to center; PlasticsOne, Roanoke, VA, USA) was then placed 0.5 mm above CA1 (AP −1.94 mm, ML ±1.25 mm, DV −1 mm) and secured to the skull using dental cement (C&B metabond, Parkell, Edgwood, NY, USA). To inhibit neuronal activity, green light (561 nm) was bilaterally delivered through two 300 μm thick optic fibers (Thorlabs, Newton, NJ, USA) that were inserted through the guide cannulas, with a 0.5 mm projection. Control mice were either uninfected with eNpHR3.1 but still implanted with the cannula delivering light into CA1, infected with eNpHR3.1 and implanted but connected to a dummy fiber that terminated the light delivery at the surface of the brain, or infected with YFP and implanted with a cannula and exposed to light. Control mice therefore experienced identical visual cues and contextual information as the experimental mice associated with laser light delivery. For BLA optogenetic inhibition, 1.5 μl of AAV5 CaMKIIα::eNpHR3.0-EYFP was microinjected into both left and right BLA (AP −1.5 mm, ML ±3.47 mm, DV −5 mm). A patchcord (i.e., implantable fiberoptic lightguide or IFL, consisting of a metal ferrule, 2.5 mm in diameter with a 200 μm thick, 5 mm long, cleaved bare optic fiber; Doric Lenses Inc., Quebec, Canada) was then placed in each BLA (AP −1.5 mm, ML ±3.47 mm, DV −4.8 mm) and secured to the skull using dental cement. Green light was bilaterally delivered through two 200 μm thick optic fibers (Doric Lenses) that were attached to the IFL using a connecting plastic sleeve. For ACC optogenetic inhibition, 1.0 μl of AAV5 CaMKIIα::eNpHR3.0-EYFP was microinjected into both left and right ACC (AP +1 mm, ML ±0.35 mm, DV −2.2 mm). The IFL was then unilaterally placed above one ACC, as close as possible to the midline (AP +1 mm, ML ±0.2 mm, DV −1.25 mm) and secured to the skull using dental cement. Green light was delivered through a 200 μm thick optic fiber (Doric Lenses) attached to the IFL. For OB optogenetic inhibition, 1.0 μl of AAV5 CaMKIIα::eNpHR3.0-EYFP was microinjected into both left and right OB (AP +4.5 mm, ML ±0.75 mm, DV −3.25 and −2 mm). An IFL was then unilaterally placed above one OB, as close as possible to the midline (AP +4.5 mm, ML ±0.15 mm, DV −1.4 mm) and secured to the skull using dental cement. Green light was delivered through a 200 μm thick optic fiber attached to the IFL.

Immunohistochemistry

Mice were anesthetized and perfused transcardially with cold PBS followed by 4% paraformaldehyde (PFA) in PBS, and brains were removed and post-fixed in 4% PFA for 3 hr at 4°C, then equilibrated in 30% sucrose in PBS. Forty micrometer-thick frozen coronal sections were stored in 25% glycerol and 30% ethylene glycol, in PBS at 4°C. Free-floating sections were washed in PBS, incubated for 30 min in 0.2% Triton X-100 (Tx100) and 2% normal donkey serum (NDS), and then incubated overnight with primary antibody in 2% NDS (mouse anti-CaMKIIα 1:500, Abcam, Cambridge, MA, USA; Rabbit anti-c-Fos 1:500, EMD Darmstadt, Germany). Sections were then washed with PBS and incubated for 2 hr at room temperature (RT) with secondary antibodies (donkey anti-mouse or donkey anti-rabbit conjugated to Cy3, 1:1000, Jackson Laboratories, West Grove, PA, USA). Slices were then washed, incubated with DAPI (1:50,000), and mounted on slides with PVA-Dabco (Sigma). Confocal fluorescence images were acquired on a scanning laser microscope using 5× or a 10× air objectives or a 40× oil immersion objective.

In Vivo Optrode Recording

Simultaneous optical stimulation and electrical recording in CA1 of mice anesthetized with isoflurane were carried out as described previously (Gradinaru et al., 2007) using an optrode consisting of an extracellular tungsten electrode (1 MΩ, ~125 μm) tightly bundled with an optical fiber (300 μm core diameter, 0.37 NA), with the tip of the electrode protruding slightly beyond the fiber end (~0.4 mm) to ensure illumination of the recorded neurons. Recordings were conducted with the optrode initially placed at the boundary of CA1 (AP −1.94 mm, ML 1.4 mm, DV −1.1 mm) and gradually lowered in 0.1 mm increments. The optical fiber was coupled to a 561 nm solid-state laser diode with ~20 mW of output from the fiber. Signals were recorded and band-pass filtered at 300 Hz low/5 kHz high using an 1800 Microelectrode AC Amplifier.

Measurement of Learning and Memory in the Fear Conditioning Paradigm

The FC apparatus consisted of a conditioning cage (18 × 18 × 30 cm) with grid floor wired to a shock generator surrounded by an acoustic chamber (Coulbourn instruments, PA, USA). To induce fear conditioning, mice were placed in the cage for 120 s, and a pure tone (2.9 kHz) was then sounded for 20 s, followed by a 2 s foot shock (0.5 mA for recent memory studies, 1 mA for remote memory studies). This procedure was then repeated, and 30 s after the delivery of the second shock, mice were returned to their home cage. FC was assessed

by a continuous measurement of freezing (complete immobility), the dominant behavioral fear response (Fanselow, 2000). Freezing was measured continuously throughout the testing trial by an experienced experimenter blind to the treatment group. To test contextual FC, mice were placed in the original conditioning cage, and freezing was measured for 5 min. To test auditory-cued FC, mice were placed in a different context (a pyramid-shaped cage with a smooth floor), freezing was measured for 2.5 min in this new cage, and then a 2.9 kHz tone was sounded for 2.5 min, during which conditioned freezing was measured. This paradigm was applied under different light conditions in the different experiments as described in the Extended Experimental Procedures. The results of contextual- and cued-conditioning tests were analyzed by Student's t test or two-way ANOVA, followed by post-hoc tests, as applicable.

Drug Delivery

For the pharmacological experiments (Figures 3E and 3F), mice were implanted with a double cannula above CA1. The cannula, surgical procedure, and location were the same as in the light delivery experiments. As described by Kitamura et al. (2009), TTX (Sigma, 20 μM) and CNQX (Tocris Bioscience, Ellisville, MO, USA; 3 mM) or saline were infused in a volume of 1 μl through a 28 gauge stainless steel internal cannula (PlasticsOne) that was 0.5 mm longer than the guide cannula. The internal cannula was connected to a micro-syringe pump (Harvard Apparatus, Holliston, MA, USA) by a PE20 tube. Solutions were administered at a constant rate of 200 nl/min, and the injection cannula was removed 2 min following the termination of the injection to avoid spillage from the guide cannula.

Open Field Test

The open field test was conducted in an open plastic arena (50 × 50 × 40 cm). Mice were placed in the center of the chamber and allowed to freely explore for 3 min. Activity in the center and periphery of the field was measured using an automated video-tracking system (Biobserve, Bonn, Germany). Percentage of time in center is defined as the percent of total time that was spent in the central 35 × 35 cm area of the open field.

Electrophysiological Measurement of Continuous Inhibition of Evoked Spiking by eNpHR3.1

Four mice from the prolonged light exposure experiment were injected, memory-tested, and then sacrificed and sliced for physiology. Coronal slices containing dorsal CA1 were prepared by perfusing ice-cold sucrose solution transcardially and cutting 300 micron slices in the same ice-cold sucrose solution. Electrophysiological recordings were made under constant perfusion of aCSF. All recordings were performed at 32°C. The patch electrode resistance was 2–6 MΩ, and series resistance was usually 10–20 MΩ. The membrane potential was corrected for a measured liquid junction potential of 7 mV. Induction of action potentials was conducted by injecting ~200 pA currents at 10 Hz. Light for the activation of eNpHR3.1 was delivered using the X-Cite 120W halogen light source through a 531 ± 20 nm filter and a 40×/0.8 NA water objective at 7 mW/mm^2. See Extended Experimental Procedures for solutions.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, three figures, and two movies and can be found with this article online at doi:10.1016/j.cell.2011.09.033.

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