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Current Biology

Recovery of "Lost" Infant Memories in Mice

Highlights

- Infant, but not adult, mice forget contextual fear memories (infantile forgetting)
- Stimulation of dentate gyrus encoding ensembles recovers lost memories in adulthood
- Memory recovery was observed up to 3 months following training
- Memory recovery was associated with reactivation of hippocampal and cortical neurons

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In Brief

Infancy is associated with rapid forgetting of event-related memories. Guskjolen et al. find that direct stimulation of encoding neuronal ensembles is sufficient to recover these lost memories in adulthood.

Guskjolen et al., 2018, Current Biology *28*, 1–8
July 23, 2018
https://doi.org/10.1016/j.cub.2018.05.059

CellPress

Please cite this article in press as: Guskjolen et al., Recovery of "Lost" Infant Memories in Mice, Current Biology (2018), https://doi.org/10.1016/j.cub.2018.05.059

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Recovery of "Lost" Infant Memories in Mice

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https://doi.org/10.1016/j.cub.2018.05.059

SUMMARY

Hippocampus-dependent, event-related memories formed in early infancy in human and non-human animals are rapidly forgotten. Recently we found that high levels of hippocampal neurogenesis contribute to accelerated rates of forgetting during infancy. Here, we ask whether these memories formed in infancy are permanently erased (i.e., storage failure) or become progressively inaccessible with time (i.e., retrieval failure). To do this, we developed an optogenetic strategy that allowed us to permanently express channelrhodopsin-2 (ChR2) in neuronal ensembles that were activated during contextual fear encoding in infant mice. We then asked whether reactivation of ChR2-tagged ensembles in the dentate gyrus was sufficient for memory recovery in adulthood. We found that optogenetic stimulation of tagged dentate gyrus neurons recovered "lost" infant memories up to 3 months following training and that memory recovery was associated with broader reactivation of tagged hippocampal and cortical neuronal ensembles.

INTRODUCTION

In humans, autobiographical memories for events that occurred early in life are forgotten more rapidly than memories for events occurring later in life, a phenomenon known as childhood or infantile amnesia [1]. This rapid forgetting has been attributed to the fact that children lack the cognitive tools to successfully consolidate and organize autobiographical memories at this early developmental stage (e.g., language [2], a sense of self-identity [3], or theory of mind [4]). However, similar accelerated forgetting in infancy is also observed in non-human species [5], including mice [6], suggesting that a complete neurobiological account cannot be limited to purely human phenomena.

We previously showed that high levels of postnatal hippocampal neurogenesis contribute to this accelerated forgetting in infant mice [7]. New neurons are generated in the subgranular zone of the dentate gyrus, and these new neurons gradually integrate into hippocampal circuits. As they integrate, they form new connections and potentially eliminate existing connections, promoting memory clearance [7–11]. Indeed, suppressing hippocampal neurogenesis in infant mice slowed forgetting of contextual fear memories, suggesting a causal relationship between neurogenesis-mediated remodeling of hippocampal circuits and forgetting during infancy [7]. However, whether remodeling leads to overwriting and memory erasure (i.e., storage failure) or simply decreases memory accessibility (i.e., retrieval failure) is unclear.

To address this issue, we developed an optogenetic strategy that allowed us to manipulate the activity of dentate gyrus neural ensembles that were activated during encoding in infancy and to test whether reactivation of these "encoding" neuronal ensembles leads to recovery of otherwise "lost" infant memories in adulthood. Infant mice trained at postnatal day 17 (P17) exhibited near-zero levels of freezing when replaced in the context 15–90 days following contextual fear conditioning. However, optogenetic stimulation of dentate gyrus neurons that were tagged with channelrhodopsin-2 (ChR2) during encoding was sufficient to induce fear memory recall across these same retention delays. Memory recovery was associated with reinstated patterns of encoding activity in the dentate gyrus and in other hippocampal subregions and the cortex (i.e., in a process akin to pattern completion).

RESULTS

Infant Mice Rapidly Forget Contextual Fear Memories

We began by characterizing infantile forgetting in wild-type (WT) mice using contextual fear conditioning. We chose contextual fear conditioning since training occurs in a single session. Furthermore, this training engages the hippocampus [7] and produces a contextual fear memory that endures for ~24 hr but then is quickly forgotten in infant mice [6, 7]. Infant (P17) and young adult (P60) WT mice were trained, and memory was tested in separate groups of mice 1–90 days later by placing mice back into the training context and assessing freezing levels (Figure 1A). Mice trained at P60 exhibited robust freezing at all delays. Indeed, freezing levels increased modestly across retention delays, likely reflecting fear incubation [12]. In contrast, mice trained at P17 exhibited forgetting, consistent with previous studies [6, 7]. Whereas freezing levels were equivalent to those

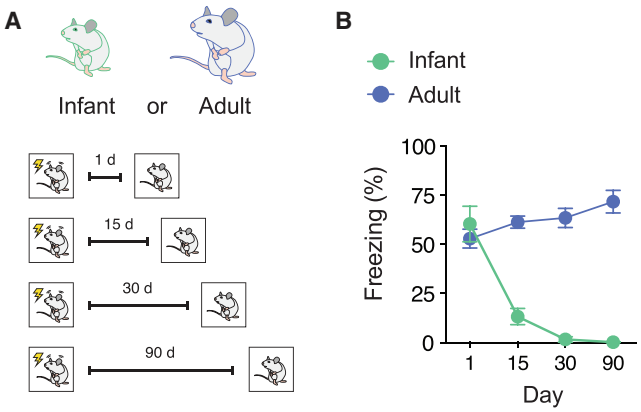

Figure 1. Infant Mice Forget Contextual Fear Memories

(A) P17 (infant) and P60 (adult) mice were trained and tested 1, 15, 30, or 90 days later.

(B) Percent freezing levels declined with retention delay in P17, but not P60, mice.

Error bars represent SEM.

observed in P60 mice when tested 1 day after training, freezing levels declined to near-zero levels at longer retention delays (Figure 1B). An ANOVA with age (P17, P60) and retention delay (1, 15, 30, and 90 days) as between-subjects variables supported these conclusions (age, $F_{1,64}$ = 135.6, p < 0.001; retention delay, $F_{3,64}$ = 10.37, p < 0.001; age × retention delay, $F_{3,64}$ = 19.12, p < 0.001).

Direct Reactivation of Dentate Gyrus Neuronal Ensembles that Were Activated during Fear Learning Induces Memory Recovery in Adulthood

We next asked whether direct reactivation of the neuronal ensembles that were activated during acquisition of contextual fear during infancy could recover these lost infant memories in adulthood. To permanently "tag" neurons active during training, we crossed mice in which the tamoxifen (TAM)-dependent recombinase Cre^{ERT2} is expressed in an activity-dependent manner from the loci of the immediate early gene *Arc* (Arc-Cre^{ERT2}, "Arc-TRAP" mice [13]), with mice expressing a floxed-stop ChR2-eYFP cassette. In offspring expressing both transgenes (Arc-ChR2 mice), cells active shortly after TAM injection permanently express a ChR2-eYFP tag, which allows these neurons to be visualized and manipulated (Figure 2A).

We trained 17-day-old Arc-ChR2 mice in contextual fear conditioning in the presence of TAM to tag active neurons. Mice were tested 15 days later (i.e., at a retention delay when significant forgetting would be anticipated [6]). As predicted, freezing levels were low in the group of mice tested without laser light stimulation (no reactivation group). In contrast, in the reactivation

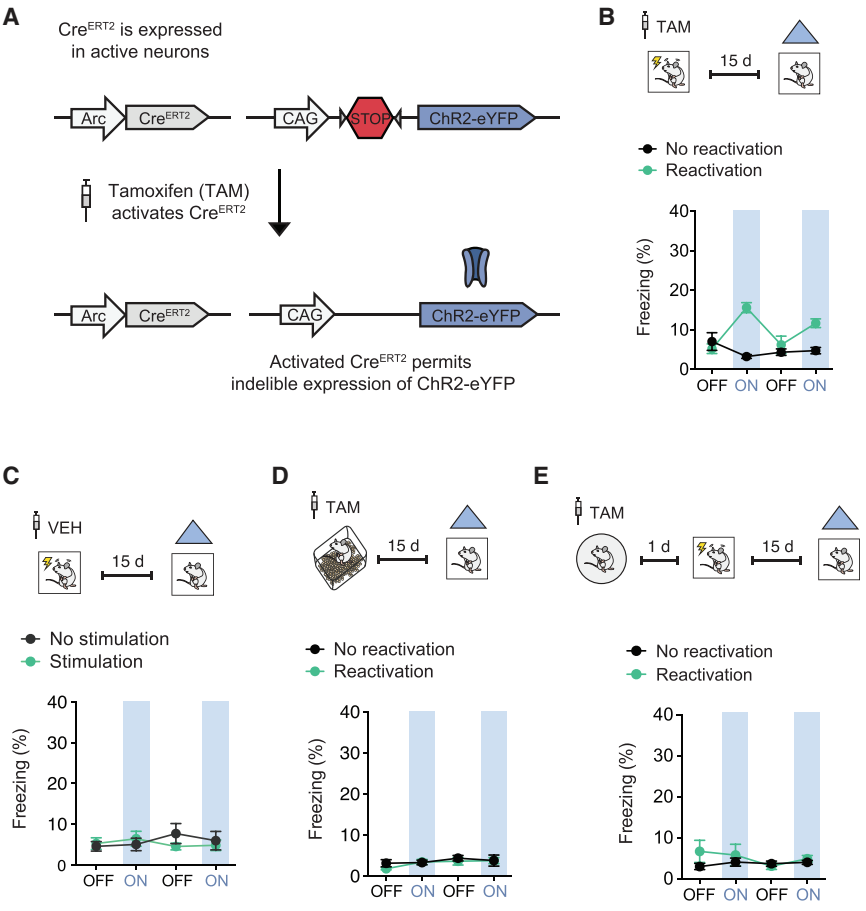

Figure 2. Optogenetic Reactivation of Encoding Ensembles in Dentate Gyrus Induces Recovery of Infant Memories

(A) Arc-TRAP mice express the TAM-dependent recombinase Cre^{ERT2} in an activity-dependent manner from the loci of the immediate early gene *Arc*. Cre-dependent recombination occurs only in active (Arc^{+}) cells following TAM treatment, permanently tagging those cells with ChR2-eYFP.

(B) In the reactivation group tested 15 days following training (filled green circles), light stimulation (blue shading) induced freezing. In contrast, in the no-reactivation group (filled black circles), in which mice did not receive light stimulation, freezing levels were low throughout the testing period.

(C) When mice were treated with VEH, not TAM, at the time of training (no-tag experiment), light stimulation did not induce freezing.

(D) When mice were treated with TAM in the home cage (home-cage experiment) and then tested, light stimulation did not induce freezing.

(E) When mice were treated with TAM in an alternate context (distinct-context experiment) and then trained and tested, light stimulation did not induce freezing.

Error bars represent SEM.

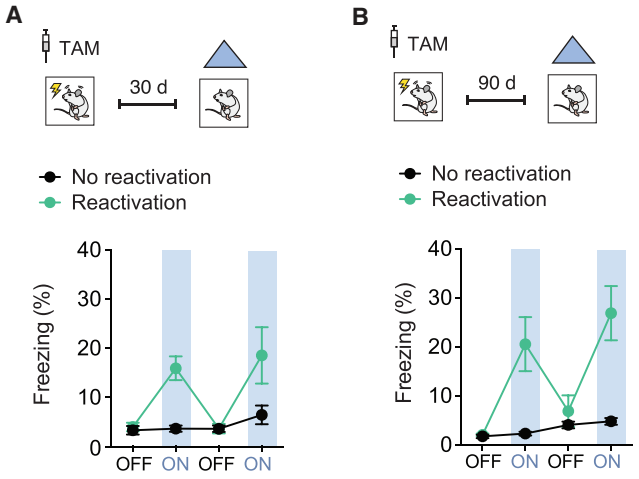

Figure 3. Optogenetic Reactivation of Encoding Ensembles in Dentate Gyrus Induces Recovery of Infant Memories, Even at Very Remote Delays

(A) In the reactivation group tested 30 days following training, light stimulation induced freezing. In contrast, in the no-reactivation condition, in which mice did not receive light stimulation, freezing levels were low throughout the testing period.

(B) In the reactivation group tested 90 days following training, light stimulation induced freezing. In contrast, in the no-reactivation condition, in which mice did not receive light stimulation, freezing levels were low throughout the testing period. Error bars represent SEM.

group, freezing levels were high in the presence of light stimulation (reactivation group, light ON epochs) but low in the periods when the light was not turned on (reactivation group, light OFF epochs) (Figure 2B). Therefore, reactivation of tagged dentate gyrus neurons led to recovery of conditioned freezing in adult mice. A mixed ANOVA with group (no reactivation, reactivation) as a between-subjects variable and time (ON, OFF) as a within-subjects variable supported this conclusion (group, $F_{1,22} = 16.54$, $p < 0.001$; time, $F_{3,66} = 4.31$, $p < 0.001$; group × time, $F_{3,66} = 11.32$, $p < 0.001$).

To evaluate the specificity of these effects, we conducted several control experiments (Figures 2C–2E). First, opto-stimulation alone (in Arc-ChR2 mice that were trained identically but treated with Vehicle [VEH] rather than TAM) did not induce freezing in the test context (mixed ANOVA: group, $F_{1,10} = 0.11$, $p = 0.75$; time, $F_{3,30} = 0.30$, $p = 0.82$; group × time, $F_{3,30} = 1.21$, $p = 0.32$) ("no tag" experiment; Figure 2C). Second, opto-stimulation of neurons tagged in the home cage (rather than in the training context) did not induce freezing in the test context (mixed ANOVA: group, $F_{1,11} = 0.38$, $p = 0.55$; time, $F_{3,33} = 2.20$, $p = 0.11$; group × time, $F_{3,33} = 0.55$, $p = 0.65$) ("home cage" experiment; Figure 2D). Third, opto-stimulation of neurons tagged in a distinct, but neutral, context (i.e., not the fear-conditioned context) the day before contextual fear training did not induce freezing in the test context (mixed ANOVA: group, $F_{1,8} = 0.73$, $p = 0.41$; time, $F_{3,24} = 0.91$, $p = 0.45$; group × time, $F_{3,24} = 1.45$, $p = 0.25$) ("distinct context" experiment; Figure 2E). The absence of freezing during opto-stimulation across these control experiments indicates that memory recovery critically depends on the combination of light stimulation and ChR2 expression and is specific to reactivation of neuronal ensembles that were active during the training episode (i.e., ensembles that presumably correspond to critical components of the engram supporting that contextual fear memory [14]).

Direct Reactivation of Dentate Gyrus Neuronal Ensembles that Were Activated during Fear Learning during Infancy Induces Memory Recovery Even at Very Remote Time Points

In our initial experiment, forgetting was most pronounced 30 and 90 days following training, with mice trained as infants exhibiting near-zero levels of freezing at these remote retention delays (Figure 1B). We next asked whether direct engram reactivation would also lead to memory recovery at these remote time points. P17 Arc-ChR2 mice were treated with TAM, trained as above, and then tested either 30 or 90 days later. At both retention delays, opto-stimulation induced freezing (Figures 3A and 3B), indicating that reactivation of dentate neuronal ensembles that were active during initial encoding was sufficient to recover memory even at very remote time points. Previous studies suggest a temporally extended role for the hippocampus in processing contextual memories [15, 16]. The current results support the view that direct stimulation of hippocampal engram neurons can lead to artificial memory expression, even at time points remote to original training [17]. Mixed ANOVAs supported the conclusions that opto-stimulation induced memory recovery at the 30 day (group, $F_{1,15} = 6.88$, $p < 0.05$; time, $F_{3,45} = 8.72$, $p < 0.001$; group × time, $F_{3,45} = 5.14$, $p < 0.05$) and 90 day (group, $F_{1,11} = 19.64$, $p < 0.001$; time, $F_{3,33} = 7.49$, $p < 0.001$; group × time, $F_{3,33} = 5.80$, $p < 0.05$) delays.

In these experiments, we used male and female mice. There were no sex-dependent differences in rates of memory recovery (data not shown). While there was a trend toward higher levels of freezing following opto-stimulation in mice tested at longer retention delays (e.g., compare Figure 3B to Figure 2B), these differences were not statistically reliable.

Memory Recovery Is Associated with Reinstating Patterns of Encoding Activity beyond the Dentate Gyrus

These experiments indicate that reactivation of tagged dentate gyrus granule cells is sufficient for memory recovery. The hippocampus is thought to orchestrate memory retrieval by reinstating patterns of cortical activity that were present during learning [18]. Consistent with this idea, inhibiting hippocampal neurons that were active during contextual fear conditioning prevents reactivation of neural ensembles in the cortex and impairs contextual fear memory recall [19]. Therefore, we next asked whether our focal stimulation led to memory recovery via more broadly reinstating patterns of encoding activity (e.g., in the cortex) via a pattern-completion-like process.

To do this, we quantified expression of the activity-regulated gene, *c-Fos*, in cortical and subcortical regions following fear memory testing (Figure 4). We then asked what proportion of the neurons that were tagged during training were reactivated during testing following opto-stimulation of the dentate gyrus across the various experimental conditions (i.e., $P(\text{c-Fos}^+|\text{eYFP}^+)$). We first assessed *c-Fos* expression in tagged neurons in the dentate gyrus, the target of our opto-stimulation. We found that opto-stimulation induced *c-Fos* in this population of granule cells,

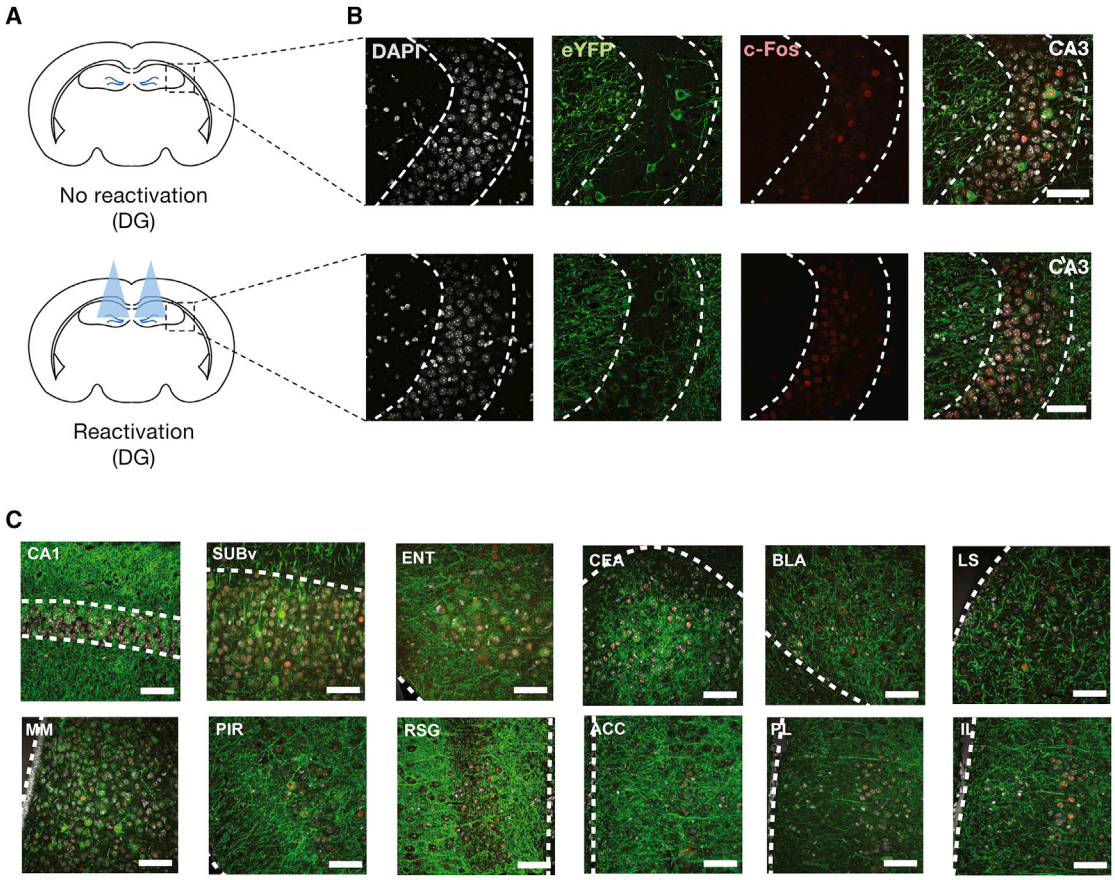

Figure 4. Characterization of Reactivation in Hippocampal and Cortical Brain Regions following Memory Recovery

(A) In each experiment, mice received either no opto-stimulation (no-reactivation group) or opto-stimulation of the dentate gyrus (DG) (reactivation group).

(B) Images showing DAPI$^+$, eYFP$^+$, c-Fos$^+$, and c-Fos$^+$eYFP$^+$ cells in the CA3 in the no-reactivation (upper) versus reactivation (lower) groups in the experiment in which opto-stimulation induced memory recovery in mice tested 15 days following training.

(C) Example images showing DAPI$^+$ (white), Fos$^+$ (red), and eYFP$^+$ (green) in remaining 12 brain regions analyzed.

CA3, CA3 field; CA1, CA1 field; SUBv, subiculum, ventral part; ENT, entorhinal cortex; CEA, central amygdala; BLA, basolateral amygdala; LS, lateral septal nucleus; MM, medial mammillary nucleus; PIR, pirirform cortex; RSG, retrosplenial cortex; ACC, anterior cingulate cortex; PL, prelimbic cortex; IL, infralimbic cortex. Scale bar, 50 μm.

indicating that reactivation was efficient (Day 15: t_{18} = 7.97, p < 0.001; Day 30: t_9 = 3.04, p < 0.05; Day 90: t_7 = 14.57, p < 0.001; home cage: t_8 = 10.80, p < 0.001; distinct context: t_{10} = 10.17, p < 0.001) (Figures 5A–5E).

We next asked whether opto-stimulation in the dentate gyrus preferentially reactivated tagged neurons beyond the dentate gyrus. To do this, we examined *c-Fos* expression in both eYFP$^+$ and eYFP$^-$ populations, collapsed across all regions analyzed. These analyses revealed higher rates of activation in tagged (eYFP$^+$) versus non-tagged (eYFP$^-$) populations, and this was observed predominantly in the reactivation conditions. These observations were supported by mixed ANOVAs with group (no reactivation, reactivation) as a between-subjects variable and population (eYFP$^+$, eYFP$^-$) as a within-subjects variable, with main population effects in the 15 day ($F_{1,18}$ = 32.28, p < 0.001), 30 day ($F_{1,9}$ = 41.60, p < 0.001), 90 day ($F_{1,7}$ = 7.17, p < 0.001) delays and in the home cage ($F_{1,8}$ = 4.63, p = 0.063) and distinct context ($F_{1,18}$ = 19.28, p < 0.05) experiments. Additionally, significant population × group interactions for the mice tested at the 15 day ($F_{1,18}$ = 9.22, p < 0.001), 30 day ($F_{1,9}$ = 6.26, p < 0.05), and 90 day ($F_{1,7}$ = 15.93, p < 0.05) delays support the idea for more pronounced reactivation of eYFP$^+$ cells when opto-stimulation is delivered (and memory recovery occurs) (Figures 5F–5J). We also note that in the no-reactivation condition, there was a trend for higher rates of c-Fos expression in tagged (eYFP$^+$) versus non-tagged (eYFP$^-$) populations (although none of these contrasts reached statistical significance). This may reflect real but nonetheless subthreshold levels of reactivation associated with unsuccessful memory retrieval.

The above analyses indicated that memory recovery was associated with reactivation of tagged neurons within and beyond the dentate gyrus. In order to understand which brain regions contribute most to this effect, we next conducted partial least-squares (PLS) analysis of P(c-Fos$^+$|eYFP$^+$) across regions in the reactivation versus no-reactivation conditions. These analyses identified latent variables that maximally differentiated the reactivation versus no-reactivation conditions at the 15 day, 30 day, and 90 day retention delays (Ps < 0.05). Bootstrap ratios (or saliences) from the PLS analyses were used to determine the extent to which reactivation rates in

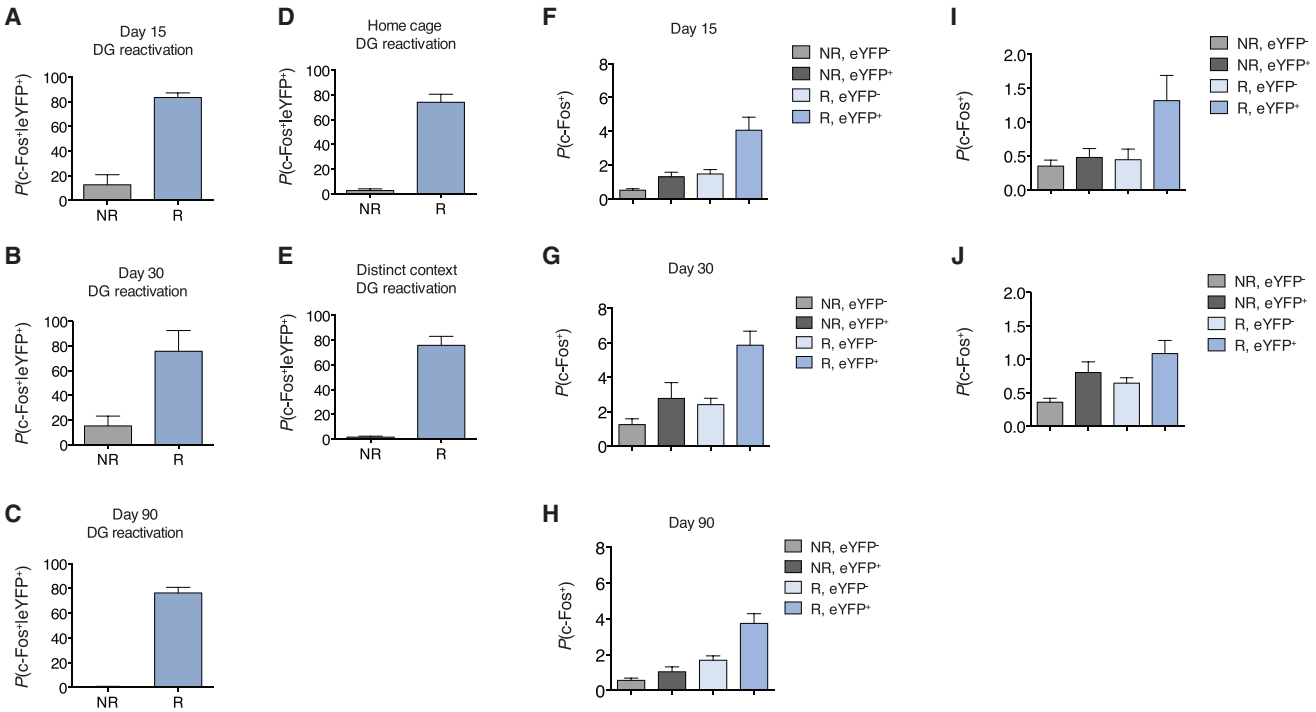

Figure 5. Stimulation of Dentate Gyrus Neuronal Encoding Ensembles Preferentially Reactivates Tagged Ensembles Brain-wide

(A–E) Dentate gyrus opto-stimulation reactivates tagged (eYFP$^+$) granule cells in the dentate gyrus. Reactivation probability (P(c-Fos$^+$|eYFP$^+$)) for no-reactivation (NR) versus reactivation (R) groups across the five experiments (15 day retention [A], 30 day retention [B], 90 day retention delay [C], home cage [D], and distinct context [E]). Reactivation rates ranged from 74% to 83%.

(F–J) Dentate gyrus opto-stimulation preferentially reactivates tagged (eYFP$^+$) versus non-tagged (eYFP$^-$) neurons across all brain regions. Activation probability (P(c-Fos$^+$) for no-reactivation (NR) versus reactivation (R) groups across the five experiments (15 day retention [F], 30 day retention [G], 90 day retention delay [H], home cage [I], and distinct context [J]).

Error bars represent SEM.

individual brain regions contributed to the ability to distinguish the reactivation versus no-reactivation groups. Bootstrap ratios are equivalent to z scores and thresholds ranging from 2.57 to 3.00 correspond to p values ranging from 0.01 to 0.0027 [20, 21]. We chose a commonly used conservative threshold of 3.00 [22–24] to identify regions contributing to significant contrasts, and this threshold is shown in red in the graphs illustrated in Figure 6.

At the 15 day delay, distinct patterns of *c-Fos* expression in the reactivation versus no-reactivation conditions was primarily driven by reactivation of neuronal ensembles in the hippocampus (i.e., CA3, CA1, ventral subiculum) (Figures 6A and S1). At longer retention delays, memory recovery was additionally associated with activation of some cortical regions (including entorhinal and piriform cortices; Figures 6B–6C, S2, and S3). This suggests that focal stimulation of tagged dentate gyrus neurons induces memory recovery by broadly reinstating patterns of encoding activity both within and beyond the dentate gyrus [25]. In contrast, in the control experiments, in which optogenetic stimulation did not lead to memory recovery, reinstatement was not observed. In these cases, *c-Fos* induction in neurons tagged in either the home cage or a distinct, but neutral, context (the day prior to contextual fear conditioning) were not differentiated by PLS analysis in the reactivation and no-reactivation conditions (Ps > 0.05) (Figures 6D–6E, S4, and S5).

DISCUSSION

Accelerated forgetting of hippocampus-dependent, event-related memories during infancy is observed in many species, including humans. During this developmental period, neurogenesis persists at high rates in the hippocampus, and the integration of newly generated neurons continuously remodels hippocampal circuits. We previously causally linked these two phenomena, showing that the high levels of hippocampal neurogenesis contribute to these accelerated forgetting rates [7]. Here, we developed an optogenetic strategy that allowed us to ask whether it is possible to recover lost infant memories during adulthood. We find that direct optogenetic stimulation of neuronal ensembles in the dentate gyrus that were active during formation of contextual fear memories induced successful memory recall during adulthood. Memory recovery was observed up to 90 days following training and was associated with reinstatement of patterns of encoding activity both within the dentate gyrus and beyond.

In these experiments, memory loss in infant mice appeared to be profound. Mice, trained as infants, exhibited near-zero levels of freezing when replaced in the original training context at later time points. However, we found that opto-stimulation of neural ensembles that were engaged during training was sufficient to induce conditioned freezing at the same retention delays.

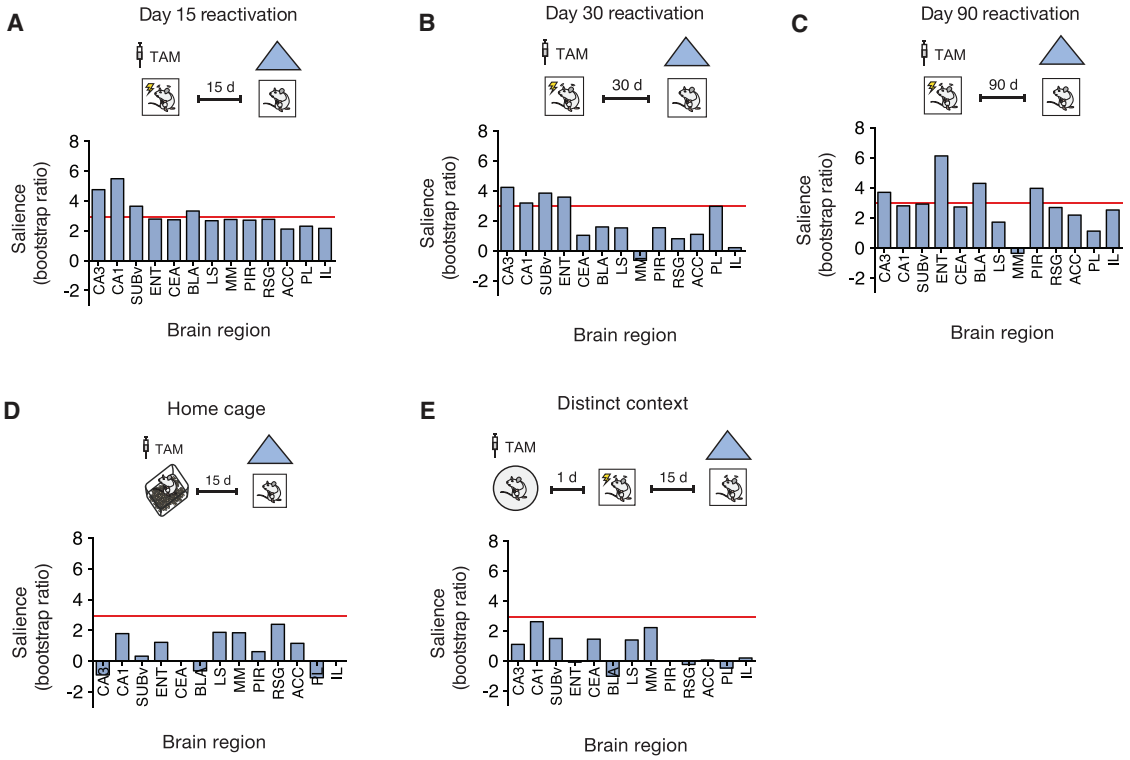

Figure 6. Memory Recovery Is Associated with Reinstatement of Patterns of Encoding Activity beyond the Dentate Gyrus

(A–E) Task PLS analysis revealed different patterns of reinstatement (i.e., $P(\text{c-Fos}^{+}|\text{eYFP}^{+})$) in the reactivation versus no-reactivation groups at the 15 day ($p = 0.003$), 30 day ($p = 0.015$), and 90 day ($p = 0.015$) delays. In contrast, similar analyses did not differentiate reactivation versus no-reactivation groups in the two control experiments (home cage, $p = 0.16$; distinct context, $p = 0.33$). Salience scores (bootstrap ratios) indicate the extent to which regions reliably distinguish reactivation versus no-reactivation groups. Salience scores larger than ± 3 (red lines) consistently differentiated reactivation versus no-reactivation groups during resampling (bootstrapping).

CA3, CA3 field; CA1, CA1 field; SUBv, subiculum, ventral part; ENT, entorhinal cortex; CEA, central amygdala; BLA, basolateral amygdala; LS, lateral septal nucleus; MM, medial mammillary nucleus; PIR, pirirform cortex; RSG, retrosplenial cortex; ACC, anterior cingulate cortex; PL, prelimbic cortex; IL, infralimbic cortex.

See also Figures S1–S5.

These results suggest that the underlying engram corresponding to the fear conditioning event is not completely overwritten. Rather, this engram presumably exists in an otherwise inaccessible, dormant state, in which "natural" reminders (such as exposure to the training context) most often do not induce successful reactivation (but see [26, 27]). This pattern of results is reminiscent of other amnestic states, including mouse models of retrograde amnesia [28] and Alzheimer's disease [29], in which opto-stimulation of tagged encoding ensembles (but not presentation of natural cues alone) permits memory recovery. These findings, therefore, indicate that infantile forgetting is due, at least in part, to retrieval failure. They imply that original patterns of strengthened connections associated with these "silent" engrams are not necessarily lost but that at least a subset are weakened with time. Direct stimulation of the engram (in combination with re-exposure to the training context) may reinstate these weakened connections, leading to memory recovery [25].

However, recovery of infant contextual fear memories at all retention delays was incomplete. In adult mice, trained identically, freezing levels ranged 61%–72% in tests 15–90 days following training (Figure 1B). In infant mice, freezing levels ranged 14%–24% following opto-stimulation at these same retention delays (during light ON epochs). Three possibilities might account for incomplete memory recovery. First, successful memory recall likely requires that retrieval cues (whether internally or externally generated) reinstate patterns of activity that occurred at the time of encoding [30]. From this perspective, incomplete recovery is perhaps not surprising given the likely mismatch between the pattern of activity evoked by opto-stimulation of tagged dentate gyrus neurons (i.e., synchronous firing) and the patterns of activity evoked by natural reminder cues.

Second, in adult mice, the neural systems supporting expression of contextual fear memories change with time [31], with recall of remote contextual fear memories associated with broad activation of the cortex [24]. In contrast, less cortical re-engagement was observed here following opto-stimulation of the dentate gyrus in mice trained as infants. This may reflect less efficient pattern-completion-like processes in the cortex under these artificial conditions. However, this also likely reflects qualitative differences in how infant versus adult contextual fear memories are consolidated. Indeed, whereas adult contextual fear memories are successfully consolidated over the course of weeks, equivalent infant memories are being actively forgotten during this period and therefore perhaps not

successfully consolidated in the cortex (although, presumably, a trace remains in the hippocampus). From this perspective, opto-stimulation of tagged dentate gyrus ensembles leads to recovery of an engram that is qualitatively different (and likely impoverished) compared to the equivalent representation in adult animals.

Third, incomplete recovery also raises the possibility that some components of the original memory trace are degraded with time. For example, as neurogenesis-mediated changes accumulate, connections may be eliminated in addition to being weakened [32, 33]. In this scenario, even optimal patterns of input activity to the dentate gyrus would be insufficient for complete memory recovery. Given all these considerations, we suggest that infantile forgetting most likely reflects a mixture of storage and retrieval failure.

In previous studies of infantile forgetting in rodents, reminders or pharmacological interventions were used to recover lost infant memories [26, 27]. In these studies, however, successful memory recovery was only observed at relatively short retention delays. For example, in rats, administration of a $GABA_A$ inverse agonist prior to testing 10 days, but not 60 days, following contextual fear conditioning training induced memory recovery [26]. In our studies, we observed recovery at considerably longer delays—up to 90 days following training. In this case, it is possible that the combination of natural cues (i.e., context re-exposure) and direct stimulation of encoding neuronal ensembles drives activation levels within the dentate gyrus (as well as in downstream target regions) beyond the threshold required for successful pattern completion. Our *c-Fos* analyses support this idea. Only modest reactivation of tagged neurons was observed in mice simply placed back in context (no-reactivation group YFP^+ versus YFP^- cells; Figures 5A–5C). In contrast, much higher levels of brain-wide reactivation were observed in mice placed in the context and additionally stimulated (reactivation group, YFP^+ versus YFP^- cells; Figures 5A–5C).

One curious finding is that memory recovery did not persist into the light OFF periods. This pattern has been observed in many similar studies following reactivation of tagged engram cells in the dentate gyrus (e.g., [14, 17, 25, 28, 29, 34]). Our study differs in one important regard from some of these previous papers in so far as reactivation occurred in the original training context—that is, direct engram stimulation occurs in the presence of natural cues (the context). One possibility is that direct stimulation of tagged dentate gyrus neurons reinstates only mossy fiber-CA3 connections (and potentially downstream targets) but not inputs into the dentate gyrus (e.g., via perforant path) or sensory cortical regions further upstream. In this case, in light OFF periods, these weaker upstream connections are not sufficient to drive dentate gyrus activation and pattern completion further downstream.

Similar to many other studies, in the current study we targeted the dentate gyrus. While artificial memory expression has been observed following opto-stimulation of tagged neurons in other brain regions including CA1 [28], retrosplenial cortex [35] and anterior cingulate cortex [17], nonetheless the dentate gyrus appears to be a hotspot for these effects. The most likely reason why stimulation of tagged neurons in the dentate gyrus is effective in these sorts of studies is because it is directly upstream of CA3, and strong recurrent excitation within CA3 efficiently drives pattern completion (leading to successful memory retrieval) [36]. Consistent with this, we found that tagged CA3 cells were reliably reactivated in all cases where memory recovery was observed.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- CONTACT FOR REAGENT AND RESOURCE SHARING
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  - Confocal microscopy
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at https://doi.org/10.1016/j.cub.2018.05.059.

ACKNOWLEDGMENTS

These experiments were supported by Canadian Institute of Health Research (CIHR) grants to S.A.J. (MOP74650) and P.W.F. (FDN143227). P.W.F. is a CIHR Canada Research Chair in Cognitive Neurobiology. S.A.J. is a CIHR Canada Research Chair in Memory Function and Dysfunction. A.G. was supported by a Restracomp fellowship from the Hospital for Sick Children. J.W.K. was supported by a long-term fellowship from Human Frontiers Science Program (LT000759/2014). J.d.l.P. was supported by a fellowship from Spanish MINECO (SAF2012-33216). We thank Brandon Walters for discussion of these experiments, Leonardo Restivo for technical assistance with immunohistochemistry, and Albert Park for artwork.

AUTHOR CONTRIBUTIONS

A.G., S.A.J., and P.W.F. conceived the project and designed the experiments. A.G. conducted the behavioral experiments. A.G., J.d.l.P., and B.-r.A.Y. conducted the immunohistochemical analyses. A.G. and J.W.K. conducted the statistical analyses. P.W.F., S.A.J., and A.G. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

Received: March 19, 2018
Revised: May 9, 2018
Accepted: May 22, 2018
Published: July 5, 2018

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
chicken anti-GFP	Aves	Cat# GFP-1010; RRID: AB_2307313
rabbit anti-c-Fos	Santa Cruz	Cat# sc-52; RRID: AB_2106783
Alexa 488 goat anti-chicken	Invitrogen	Cat# A-11039; RRID: AB_142924
Alexa 568 goat anti-rabbit	Invitrogen	Cat# A-11011; RRID: AB_143157
Chemicals, Peptides, and Recombinant Proteins		
4-Hydroxytamoxifen	Sigma	CAS: 68047-06-3
4'6'-diamidino-2-phenylindole (DAPI)	Vectashield	CAS: 28718-90-3
Dimethyl sulfoxide (DMSO)	Sigma	CAS: 67-68-5
Cremaphore	Sigma	CAS: 61791-12-6
Deposited Data		
Raw and analyzed data	This paper	N/A
Experimental Models: Organisms/Strains		
Mouse: 129Svev	Taconic Bioscience	Cat# 5654129; RRID: MGI:5654129
Mouse: Arc^{CreER} (i.e., Arc-cre^{ERT2})	The Jackson laboratory	JAX: 021881; RRID: IMSR_JAX:021881
Mouse: Ai32 or Ai32(RCL-ChR2(H134R)/EYFP)	Allen brain institute, but can now be found at The Jackson Laboratory	JAX: 024109; RRID: IMSR_JAX:024109
Software and Algorithms		
Freeze frame	Actimetrics	http://www.actimetrics.com/products/freezeframe/
Fiji (ImageJ)	ImageJ	https://fiji.sc
GraphPad Prism 5	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Adobe Illustrator C6	Adobe	https://www.adobe.com/ca/products/illustrator.html
Custom software to analyze freezing	Josselyn/Frankland lab	http://cyansite.cc/freeze.html
R v3.3.3	CRAN@R-project	https://cran.r-project.org/web/packages/sna/index.html

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Paul Frankland (paul.frankland@sickkids.ca).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All procedures were conducted in accordance with policies of the Hospital for Sick Children Animal Care Committee and conformed to both Canadian Council on Animal Care (CCAC) and National Institutes of Health (NIH) Guidelines on Care and Use of Laboratory.

Experimental Animals

Two lines of mice were used. First, to evaluate forgetting rates in infant and adult mice, we used wild-type (WT) C57BL/6N mice (Taconic Farms, Germantown, NY). Second, to test whether optogenetic reactivation promotes memory recovery, we crossed transgenic mice in which tamoxifen (TAM)-dependent recombinase Cre^{ERT2} is expressed in an activity-dependent manner from the loci of the immediate early gene Arc (Arc-CreERT2, "Arc-TRAP" mice [13]) with transgenic mice expressing a floxed-stop ChR2-EYFP cassette (Ai32 or Ai32(RCL-ChR2(H134R)/EYFP; B6;129S-*Gt(ROSA)26Sor*$^{tm32(CAG-COP4*H134R/EYFP)Hze}$/J, strain 012569). In offspring expressing both transgenes (Arc-ChR2 mice), neurons in which Arc is induced shortly after TAM injection permanently express ChR2. Mouse genotypes were determined by PCR analysis of tail DNA samples.

All mice were bred in our colony at The Hospital for Sick Children. The day of birth was designated postnatal day 0 (P0). After weaning at P21, same sex mice were group-housed in standard mouse housing cages (2–5 per cage). Rooms were maintained on a 12 h light/dark cycle and behavioral testing occurred during the light phase of the cycle. Both male and female mice were used in all experiments. All procedures were approved by Hospital for Sick Children Animal Care and Use Committee and conducted in accordance with CCAC and US National Institutes of Health (NIH) guidelines.

METHOD DETAILS

Evaluation of forgetting in WT mice

We assessed forgetting in infant (P17) versus adult (P60) mice using contextual fear conditioning.

Contextual fear training

Training consisted of placing mice into the fear conditioning chamber (31 cm × 24 cm × 21 cm; Med Associates) with shock-grid floors (bars 3.2 mm diameter, spaced 7.9 mm apart). The front, top and back of the chamber were clear acrylic and the two sides were modular aluminum. Footshocks (0.5 mA, 2 s duration) were delivered 120 s, 180 s, 240 s, 300 s and 360 s after placement in the chamber. Mice were removed from the conditioning chamber 60 s following the final shock and returned to their home cage.

Memory testing

Separate groups of infant and adult mice were tested either 1 (P17, N = 9; P60, N = 7), 15 (P17, N = 9; P60, N = 12), 30 (P17, N = 10; P60, N = 7), or 90 days (P17, N = 8; P60, N = 10) after training. Testing consisted of placing mice back into the fear conditioning chamber for 5 mins. During training and testing, mouse behavior was monitored continuously by a video camera mounted on the ceiling of the fear conditioning chamber. Contextual fear memory was assessed by measuring the amount of time mice spent freezing (i.e., the absence of movement except for breathing [37]) assessed using an automated scoring system (Actimetrics).

Optogenetic memory reactivation in Arc-ChR2 mice

In this experiment, we used the Arc-ChR2 mice to 'tag' neurons in the dentate gyrus that were activated as infant mice formed a contextual fear memory, and then tested whether subsequent optogenetic reactivation of these tagged neurons promoted memory recovery [14, 17, 25, 28, 29].

Contextual fear training

Arc-Chr2 transgenic mice were fear conditioned as above.

Drug

Immediately upon removal from the fear conditioning chambers, recombination was induced in mice with via an intraperitoneal (i.p.) injection of 4-Hydroxytamoxifen (TAM; 25 mg/kg and injected at 5 μl/g mouse).

TAM powder was first mixed with 100% ethanol (15 mg/375 μl) and vortexed vigorously. The solution was then poured into a 50°C chamber, vortexing every 12 min for approximately 2 h until fully dissolved. An equal part cremaphore (375 μl) was added to create a stock solution that was stored at −20°C until required. On experimental days, the stock solution was mixed at a 1:2 ratio with PBS. Vehicle (VEH) solution was prepared identically, excluding the TAM.

Surgery

Four days before the memory test, mice were implanted bilaterally with optical fibers immediately above the dorsal dentate gyrus. For mice trained at P17, and tested 15 d later, we used the following coordinates (relative to bregma): anterior-posterior [AP] +1.3 mm, medial-lateral [ML] ± 2.0 mm dorsal-ventral [DV] −1.7mm. For mice trained at P17, and tested either 30 or 90 d later, we used the following coordinates: AP +1.5 mm, ML ± 2.2 mm, DV −1.9 mm. For surgery, mice were pre-treated with atropine sulfate (0.1 mg/kg, i.p.), anesthetized with chloral hydrate (400 mg/kg, i.p.), and then placed into stereotaxic frames. Optical fibers were constructed in-house by attaching a 10 mm piece of 200 μm, optical fiber (with a 0.37 numerical-aperture, NA) to a 1.25 mm zirconia ferrule (fiber extended 2 mm beyond ferrule). Fibers were attached with epoxy resin into ferrules, cut and polished. Optical fibers were stabilized to the skull with screws and dental cement. Dental cement was painted black to minimize light leakage. After surgery, mice were then treated with analgesic (Ketoprofen, 5 mg/kg, subcutaneous injection) and 1 mL of 0.9% saline (subcutaneous) and fitted with plastic caps over the implanted optical fibers.

Contextual fear test and optogenetic stimulation

Either 15 (no reactivation group, N = 12; reactivation group, N = 12), 30 (no reactivation group, N = 8; reactivation group, N = 9), or 90 days (no reactivation group, N = 6; reactivation group, N = 7) following fear training, mice were placed back in the training context for a 12 min memory test. For mice in the reactivation groups, there were two light stimulation ("light ON") epochs from 180-360 s and from 540-840 s (473 nm; 20 Hz, 15ms pulses, 5 vpp, 30% duty cycle, at 0.9-1.0 mW). Mice in the no reactivation conditions were tested identically, except that light stimulation was not delivered. In these optogenetic experiments, freezing was characterized as cessation of movement by an experimenter blind to the condition of the animal using customized software: http://cyansite.cc/freeze.html. In our experiments opto-stimulation targeting the dentate gyrus occurred at 20 Hz. While it is possible to tag and artificially express contextual fear memories via photo-stimulation of CA1, this has only been observed at 4 Hz, and not at 20 Hz [28]. Therefore, it seems unlikely that off-target activation of tagged CA1 neurons can account for memory recovery. However, we can not exclude the possibility that off-target activation of other regions (e.g., CA3) contributes to memory recovery in our experiments.

Control experiments

Three control experiments were conducted in order to evaluate whether memory recovery depended upon the combination of light stimulation and ChR2 expression, and on reactivation of neuronal ensembles that were active during the training episode.

'No tag' control

P17 mice were trained as above, and then treated with VEH (rather than TAM). Fifteen days later they were tested, as described above. During this test, we assessed the impact of optical stimulation on freezing behavior (no stimulation group, N = 7; stimulation group, N = 5).

'Home cage' control

In this condition, P17 mice were treated with TAM in their home cage. These mice were placed in the fear conditioning chamber 15 days later and tested, as described above. During this test, we assessed the impact of reactivating ensembles tagged in the home cage on freezing behavior (no reactivation group, N = 5; reactivation group, N = 8).

'Distinct context' control

P16 mice were placed in an alternate context (45 × 45 × 20 cm) and treated with TAM. The following day these mice were trained and then tested 15 days later, as described above. During this test, we assessed the impact of reactivating ensembles tagged during exposure to the neutral context on freezing behavior (no reactivation group, N = 6; reactivation group, N = 6).

Fixation and tissue processing

Ninety minutes after the memory test, mice were anesthetized with chloral hydrate and perfused intracardially with ice-cold 0.1 M PBS followed by 4% PFA. Brains were removed, post-fixed in 4% PFA for 24 h and stored in a 30% sucrose solution until processed further. Brains were sectioned coronally at 50 μm using a cryostat.

Sectioned tissue was washed in 0.1 M PBS, maintained in blocking solution (5% normal goat serum and 0.3% Triton X-100) for 2 h, then incubated with for chicken anti-GFP (1:500; Aves) and rabbit anti-c-Fos (1:750; Santa-Cruz) primary antibodies for 72 h at 4°C. Slices were washed (0.1M PBS) and incubated in Alexa 488 goat anti-chicken (1:500; Invitrogen) and 568 goat anti-rabbit (1:500; Invitrogen) secondary antibodies for 24 h at 4°C. Sections were counterstained with DAPI (1:10000; Sigma-Aldrich), washed (0.1M PBS), then mounted on slides and coverslipped.

Confocal microscopy

Sections were imaged on a laser confocal microscope (Zeiss LSM 710). To ensure accurate identification and quantification of triple-labeled cells, all images were taken at 40X magnification. Frame size (512 × 515 pixels), image size (212 μm x 212 μm), and pixel size (0.42 μm) were kept consistent across conditions and in each neural region examined. An experimenter blind to the condition of the mouse would take an image at approximately the same location in each region of interest, while ensuring that each image would include $eYFP^+$ neurons such that a reactivation score could be calculated. For each image, an optical z stack series was acquired with images 300 μm apart. Five to eight sections were counted for each neural region of interest within each mouse. Within each stack, the total number of $DAPI^+$, $eYFP^+$, and $c\text{-}Fos^+$ neurons were counted. The proportion of neurons 'tagged' during memory encoding was calculated as $eYFP^+$/DAPI. The proportion of neurons activated during memory recall calculated as $c\text{-}Fos^+$/DAPI. Reactivation rates (i.e., the proportion of tagged cells that were reactivated during memory recall test) were calculated as ($c\text{-}Fos^+|eYFP^+$).

QUANTIFICATION AND STATISTICAL ANALYSIS

Behavioral data were analyzed using analysis of variance (ANOVA). Post hoc (Newman-Keuls) tests were used where appropriate. Statistical significance was set at $p < 0.05$.

Cell counting data were analyzed using mean-centered task partial least-squares (PLS) implemented in R (v3.3.3) and based on Krishnan et al. (2011) and McIntosh and Lobaugh (2004) [21, 38]. PLS is a technique that uncovers optimal patterns in multi-dimensional data to differentiate experimental conditions [39]. This technique has been used in both human neuroimaging and animal immunohistochemistry experiments to both determine whether brain imaging data can distinguish experimental groups, and identify what brain regions reliably differentiate conditions [24, 39, 40]. Using singular value decomposition, PLS results in singular values and pairs of latent variables. In the context of brain imaging data, one latent variable represents a contrast that differentiates experimental groups, and the other represents brain region saliences that identify the extent to which a given region contributes to its associated contrast.

Resampling statistics were used to determine the significance and reliability of experimental contrasts and brain region saliences, respectively. For contrasts, permutation tests were performed by resampling the experimental conditions for subjects without replacement 1000 times. During each resampling, singular values from PLS analysis were calculated to generate a null hypothesis distribution for comparison to the experimental value. The reliability of brain region saliences was determined using bootstrap ratios in which subjects retained their experimental condition but were resampled with replacement 1000 times. Original saliences were divided by their bootstrap derived standard deviations to generate bootstrap ratios. Bootstrap ratios above 3 were considered reliable.

DATA AND SOFTWARE AVAILABILITY

Requests should be directed to the lead contact.