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Article

Reactivation of Neural Ensembles during the Retrieval of Recent and Remote Memory

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Summary

Background: Episodic memories are encoded within hippocampal and neocortical circuits. Retrieving these memories is assumed to involve reactivation of neural ensembles that were established during learning. Although it has been possible to follow the activity of individual neurons shortly after learning, it has not been possible to examine their activity weeks later during retrieval. We addressed this issue by using a stable form of GFP (H2B-GFP) to permanently tag neurons that are active during contextual fear conditioning.
Results: H2B-GFP expression in transgenic mice was increased by learning and could be regulated by doxycycline (DOX). Using this system, we found a large network of neurons in the hippocampus, amygdala, and neocortex that were active during context fear conditioning and subsequent memory retrieval 2 days later. Reactivation was contingent on memory retrieval and was not observed when animals were trained and tested in different environments. When memory was retrieved several weeks after learning, reactivation was altered in the hippocampus and amygdala but remained unchanged in the cortex.
Conclusions: Retrieving a recently formed context fear memory reactivates neurons in the hippocampus, amygdala, and cortex. Several weeks after learning, the degree of reactivation is altered in hippocampal and amygdala networks but remains stable in the cortex.

Introduction

Episodic and contextual memories can be retrieved months and years after they are formed. Retrieving these memories is assumed to involve reactivation of hippocampal and neocortical networks that were established during learning [1–3]. Consistent with this idea, many brain regions that are active during learning are also engaged during testing [4–7]. However, it has not been possible to determine whether the same neurons that encode memory in these regions are later reactivated during retrieval.

Fluorescence in situ hybridization (FISH) studies using the immediate early genes Arc and Homer indicate that hippocampal and neocortical neurons are reactivated when animals explore the same spatial environment twice within a 30 min period [8, 9]. Longer intervals cannot be examined with this technique because mRNA for these genes rapidly decays. To overcome this issue, we used newly engineered transgenic mice that express a long-lasting, activity-dependent form of green fluorescent protein (GFP). In these animals, activation of the c-*fos* promoter during learning leads to the expression of human histone H2B-GFP, a fusion protein that takes several weeks to degrade [10–13]. As a result, the activity of neurons labeled with H2B-GFP can be examined days and weeks after learning. A similar strategy was used to demonstrate reactivation of amygdala neurons 3 days after learning in tau-LacZ reporter mice [14].

In the current experiments, we examined memory retrieval using a context fear conditioning task that is dependent on the hippocampus, amygdala, and neocortex [3, 15, 16]. Neurons were labeled with H2B-GFP during learning and then re-examined 2 days or 2 weeks later during memory retrieval. We found a large network of tagged neurons in the hippocampus, amygdala, and neocortex that were reactivated 2 days after learning. This result suggests that memory retrieval involves reactivation of individual neurons that were engaged during learning. These same networks were not reactivated when animals were trained and tested in different environments. Two weeks after learning, the pattern of reactivation was altered in the hippocampus and amygdala but remained largely unchanged in the cortex. This finding suggests that hippocampal and amygdala circuits are modified after learning, whereas cortical networks remain stable over time.

Results

Selective and Persistent Tagging of Activated Neurons in H2B-GFP TetTag Mice

To label cells during learning, we used transgenic mice that express a long-lasting, activity-dependent form of GFP. In these mice, the tetracycline-transactivator (tTA) system for transgene regulationn was combined with the c-*fos* promoter to tag active neurons (Figure 1A). In the absence of doxycycline (DOX), activation of the c-*fos* promoter leads to expression of an H2B-GFP fusion protein, which is stable for several weeks after induction [10, 13]. In the presence of DOX, H2B-GFP expression is prevented. Figure 1B shows widespread H2B-GFP expression after context fear conditioning (top) that was confined to excitatory neurons expressing αCamKII (bottom).

We first determined whether H2B-GFP TetTag mice could be used to selectively label neurons in the hippocampus that were active during context fear conditioning. Mice fear conditioned off DOX (OFF) showed greater H2B-GFP expression than homecage (HC) control animals (main effect of group $F(1, 7) = 25.28$, $p < 0.05$). Post hoc tests (Fisher's PLSD) revealed that H2B-GFP expression was significantly elevated in CA1 and CA3 (p values < 0.05) (Figure 1C). The percentage of labeled neurons was similar to that observed in previous studies, indicating that H2B-GFP is a reliable indicator of cellular activity [17, 18]. These results are consistent with a recent paper that showed that activity-dependent labeling in fos-tTA transgenic mice recapitulates endogenous c-*fos* expression [19]. We also observed a 57% increase in the percentage of H2B-GFP-positive neurons in the DG, although

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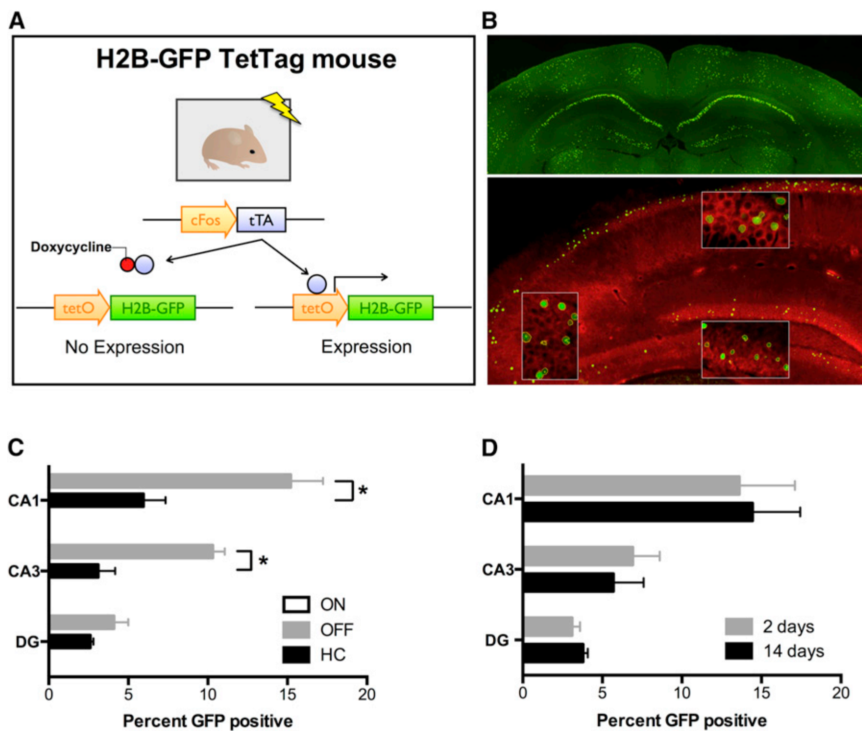

Figure 1. Selective and Persistent Tagging of Active Neurons in H2B-GFP TetTag Mice

(A) The tetracycline-transactivator (tTA) system for transgene regulation was combined with the c-*fos* promoter to tag activated neurons. In the absence of doxycycline (DOX), activation of the c-*fos* promoter leads to expression of an H2B-GFP fusion protein, which is stable for weeks after induction. In the presence of DOX, H2B-GFP expression is prevented.

(B) Robust H2B-GFP expression was observed throughout the brain in mice that underwent context fear conditioning off DOX (top). Expression of H2B-GFP was limited to excitatory neurons labeled with αCAMKII (red) in the hippocampus (bottom). The yellow outline indicates overlap between H2B-GFP-positive nuclei and cytosolic αCAMKII staining.

(C) Mice fear conditioned off DOX (OFF) (n = 4) showed greater H2B-GFP expression than homecage control (HC) animals (n = 5). H2B-GFP expression was significantly elevated in CA1 and CA3. There was a numerical increase in the percentage of H2B-GFP-positive neurons in the DG, but this change did not reach statistical significance. No H2B-GFP expression was observed in mice fear conditioned on DOX (ON) (n = 4).

(D) Two groups of mice were fear conditioned in the absence of DOX. Afterward, the animals were put back on DOX for 2 days (n = 5) or 14 days (n = 4) before being sacrificed for immunohistochemistry. We observed robust expression of H2B-GFP in DG, CA3, and CA1 that did not change over time. Error bars represent ± SEM. *p < 0.05.

this change was not statistically significant ($p = 0.1$). The lack of an effect was probably due to the small number of neurons that are typically activated in this region during learning (1%–5%) [18, 20, 21]. No H2B-GFP-positive neurons were observed in DG, CA3, or CA1 in mice that were fear conditioned on DOX (ON).

In the next experiment, we verified that the H2B-GFP signal is stable over time. TetTag mice were trained off DOX to label activated neurons with GFP. After learning, the animals were put back on DOX for 2 days or 14 days prior to brain extraction and quantification. We found equivalent H2B-GFP expression 2 days and 14 days after learning (no effect of day $F(1, 7) < 1$) (Figure 1D). These data indicate that the activity of tagged neurons can be followed for several weeks after context fear conditioning.

Reactivation of Neural Networks during Recent and Remote Memory Retrieval

We next examined reactivation of H2B-GFP neurons during memory retrieval in several brain regions (Figure 2A) 2 days and 14 days after learning (Figure 2B). Regions were analyzed in coronal sections that ranged from −2.05 mm to −2.25 mm posterior to bregma. Neurons activated during learning were labeled with H2B-GFP by removing DOX prior to training. The animals were put back on DOX immediately afterward to prevent further H2B-GFP expression. Two days or 14 days later, the mice were tested in the same context and c-*fos* expression was used to index cellular activity. H2B-GFP and c-*fos* expression were quantified in DG, CA3, CA1, and the basolateral nucleus of the amygdala (BLA). The BLA receives direct projections from the hippocampus and is essential for context fear learning and expression [22, 23]. Within the same AP coordinates, we also identified regions of interest (ROIs) in the lateral entorhinal cortex (ENTl), retrosplenial cortex (RSPv), and posterior parietal cortex (PTLp). These regions were selected based on their contributions to spatial learning and their anatomical connections with the hippocampus [23–28]. We also quantified reactivation in a control region (supplementary motor cortex [MOs]) that is not involved in context fear learning.

Figure 2C illustrates the amount of H2B-GFP expression that was observed 2 days and 14 days after learning. Consistent with our initial finding, the amount of GFP expression did not change over time (no effect of group $F(1, 7) < 1$). There was a difference in c-*fos* expression between groups (main effect of group $F(1, 7) = 32.9$, $p < 0.05$) that was limited to CA1 and RSPv (Fisher's PLSD $p < 0.05$). In these regions, the number of c-*fos*-positive neurons increased 14 days after learning (Figure 2D). To control for these differences in expression, we compared the percentage of reactivated neurons $((GFP + c-fos/DAPI) \times 100)$ to that expected by chance $((GFP/DAPI \times c-fos/DAPI) \times 100)$ in all subsequent experiments.

Previous work indicates that context fear gradually becomes independent of the hippocampus after learning [29–32]. Therefore, we next determined whether the hippocampus is required for memory retrieval 2 days and 14 days after learning. To examine this issue, we inactivated the dorsal hippocampus with the AMPAR antagonist CNQX prior to testing. Memory was assessed by measuring the freezing response, a species-specific defensive behavior observed in rodents [33]. TetTag littermates lacking H2B-GFP were trained in context A and tested 2 days or 14 days later in the same environment. Infusion of CNQX into the dorsal hippocampus

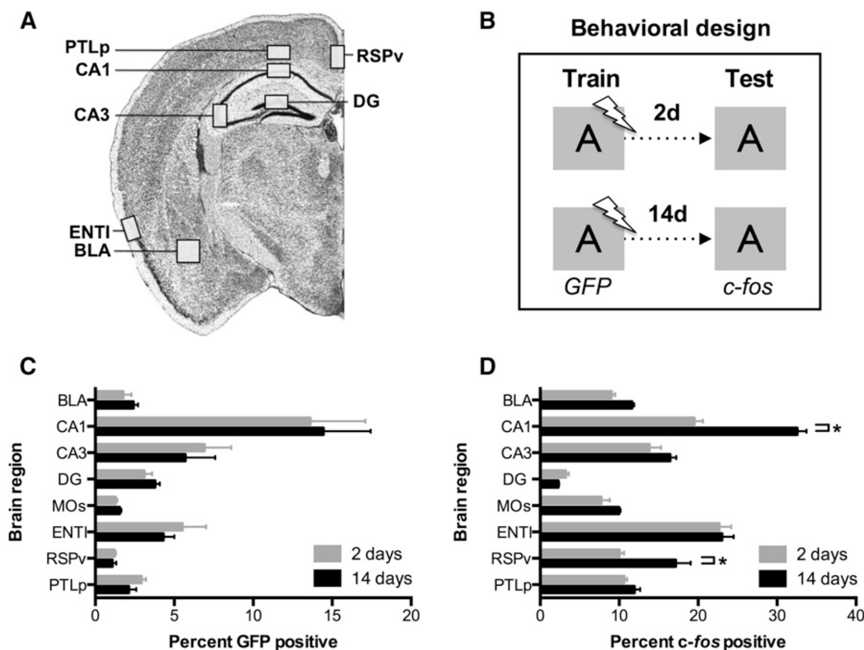

Figure 2. H2B-GFP and c-*fos* Expression after Context Fear Conditioning

(A) A coronal section (−2.05 mm posterior to bregma) illustrating the brain regions analyzed for reactivation: dorsal hippocampus (DG, CA3, CA1), lateral entorhinal cortex (ENTl), basolateral amygdala (BLA), posterior parietal association area (PTLp), and retrosplenial cortex (RSPv). Image is adapted from the Allen Reference Atlas.

(B) Behavioral procedures for the reactivation experiments. Mice underwent fear conditioning in context A off DOX to tag activated neurons with H2B-GFP. After training, animals were put back on DOX and tested 2 days (n = 5) or 14 days (n = 4) later. c-*fos* expression during testing was used to identify activated neurons. Neurons double labeled with GFP and c-*fos* were activated during training and testing.

(C) The percentage of neurons expressing H2B-GFP was equivalent 2 days and 14 days after training.

(D) c-*fos* expression was the same in most brain regions at 2 days and 14 days. It differed in CA1 and RSPv in which expression was increased 14 days after training. Error bars represent ± SEM. *p < 0.05.

prior to testing impaired memory retrieval at 2 days (main effect of group $F(1, 24) = 10.23$, $p < 0.05$) but had no effect 14 days after training (no effect of group $F(1, 22) < 1$) (Figure 3A). These results suggest that the systems mediating context fear are reorganized within 2 weeks of learning.

Given that the hippocampus is required to retrieve recent context fear memories, we expected to see reactivation of neurons in this region 2 days after learning. Figure 3B shows the percentage of reactivated neurons in TetTag mice relative to chance (chance = percent GFP × percent c-*fos*). Reactivation was significantly greater than chance in the DG, CA1, and BLA (Fisher's PLSD, pairwise comparisons, all p values < 0.05). Reactivation did not exceed chance in CA3 ($p > 0.05$). These results suggest that memory retrieval involves reactivation of neurons in the hippocampus and amygdala that were previously activated during learning.

We next determined whether the same brain regions were reactivated when memory was retrieved 2 weeks after learning. Mice were trained as described above and then tested 14 days later. Analysis of double labeling revealed that neurons in CA3 and CA1 were significantly reactivated during remote memory retrieval (Fisher's PLSD, pairwise comparisons, p values < 0.05). In contrast, reactivation in the BLA and DG did not exceed chance (p values > 0.05) (Figure 3C). To determine whether there were changes in the number of reactivated neurons over time, we compared the percentage of H2B-GFP-positive cells colabeled with c-*fos* $(GFP + c - fos/Total\ GFP)$ during the recent and remote memory tests (Figure 3D). Planned comparisons (Fisher's PLSD) found a significant reduction in the percentage of reactivated neurons in the BLA and DG during the remote memory test (p values < 0.05), an increase in CA3 ($p < 0.05$), and no change in CA1 ($p > 0.05$).

These results deviated from our predictions about remote memory reactivation in two ways. First, we did not expect to find reactivation of hippocampal neurons given that this region was not required for memory retrieval 14 days after learning. The fact that CA3 and CA1 were reactivated suggests that the hippocampus normally contributes to memory retrieval but that other structures can compensate when this region is inactivated. Recent studies are consistent with this idea [30, 34, 35]. Second, we did not observe reactivation of the BLA even though the amygdala is required for the retrieval of fear memories months and years after learning [36, 37]. Examination of our c-*fos* expression data (Figure 2D) indicates that activity in the amygdala at 2 weeks (9.9%) did not differ from that observed during memory retrieval at 2 days (7.6%) (no effect of time $F(1, 7) = 1.31$, $p > 0.05$). Therefore, it is possible that amygdala activity is required for remote memory retrieval even though reactivation of the same neurons that were engaged during learning is not. Alternatively, a smaller population of reactivated neurons (which did not exceed chance in the current experiment) may be sufficient to support memory retrieval at remote time points. To test the latter possibility, we examined the relationship between BLA reactivation and freezing. There was a strong linear relationship between the percentage of reactivated neurons and the amount of freezing at 2 days ($r^2 = 0.81$) and 2 weeks ($r^2 = 0.62$) that did not differ (no effect of time, $F(1, 5) = 3.14$, $p > 0.05$). This suggests that reactivation of BLA neurons is related to the amount of freezing during recent and remote memory retrieval. This finding is consistent with a recently published paper that used a similar genetic system to examine reactivation of amygdala neurons after fear conditioning [14].

We next examined reactivation of cortical areas that are involved in spatial and contextual learning. Figure 4A shows the percentage of reactivated neurons in each region relative to chance (chance = percent GFP × percent c-*fos*). Reactivation was significantly greater than chance in the ENTl, RSPv, and PTLp (Fisher's PLSD, pairwise comparisons, all p values < 0.05). As expected, reactivation did not exceed chance in MOs ($p > 0.05$). These results indicate that retrieval of a recently formed context fear memory involves widespread reactivation of cortical neurons that were engaged during learning.

Finally, we examined reactivation of cortical regions when memory was retrieved 2 weeks after learning. Figure 4B shows

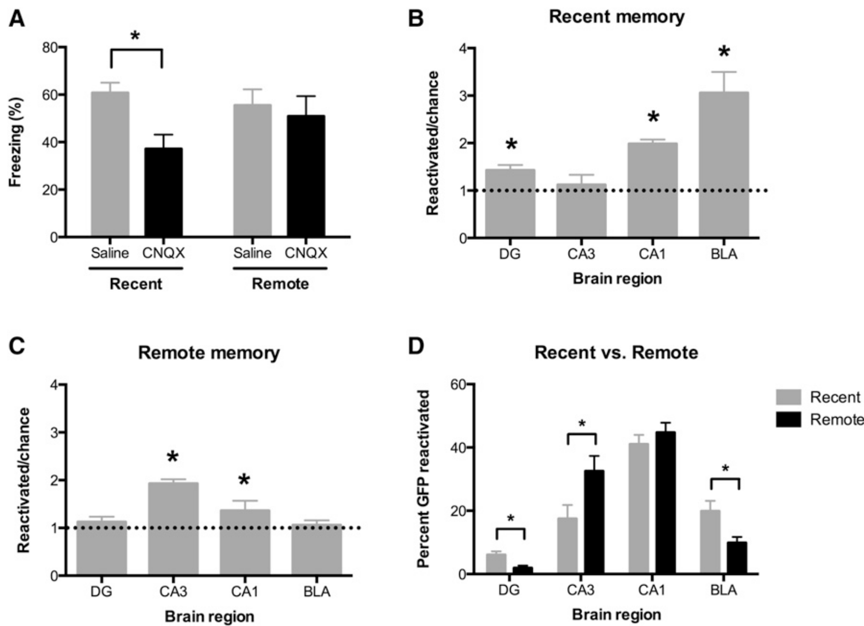

Figure 3. Reactivation of Neurons in the Hippocampus and Amygdala during Memory Retrieval

(A) Control mice were trained in context A and tested 2 days (recent, saline n = 13, CNQX n = 13) or 14 days (remote, saline n = 12, CNQX n = 12) later in the same environment. Infusion of CNQX into the dorsal hippocampus prior to testing impaired memory retrieval at 2 days but not 14 days after training.

(B) TetTag mice were trained off DOX in context A and tested in the same environment 2 days later (n = 5). The percentage of double-labeled neurons is shown relative to chance (percent H2B-GFP × percent c-*fos*). Significant reactivation was observed in DG, CA1, and BLA but not CA3.

(C) A separate group of TetTag mice were trained in context A and tested in the same environment 14 days later (n = 4). Significant reactivation was observed in CA3 and CA1 but not in DG or BLA.

(D) The percentage of H2B-GFP-positive neurons that were reactivated (i.e., colabeled with c-*fos*) is shown for mice tested 2 days (recent, n = 5) or 14 days (remote, n = 4) after training. The percentage of reactivated neurons decreased over time in the BLA and DG, increased in CA3, and remained unchanged in CA1. Error bars represent ± SEM. *p < 0.05.

the percentage of reactivated neurons in each region relative to chance. We found that reactivation was significantly greater than chance in the ENTl and PTLp (Fisher's PLSD, pairwise comparisons, p values < 0.05) but not in the RSPv or MOs (p values > 0.05). To determine whether there were changes in the number of reactivated neurons over time, we compared the percentage of H2B-GFP-positive cells colabeled with c-*fos* $(GFP + c - fos/Total\ GFP)$ during the recent and remote memory tests (Figure 4C). This analysis revealed that the percentage of reactivated neurons did not change over time in any of the cortical regions examined (all p values > 0.05).

Reactivation in RSPv did not exceed chance 14 days after learning because of an increase in c-*fos* expression (illustrated in Figure 2D), not because of a decline in the percentage of reactivated neurons. This result is consistent with a previous study that found increased expression of c-*fos* in the retrosplenial cortex during the retrieval of remote spatial memories [26]. As shown in Figure 2D, we did not observe a change in c-*fos* expression over time in any of the other cortical regions examined. Therefore, our data suggest that reactivation of cortical networks remains relatively stable after learning.

Memory Retrieval Is Required for Reactivation of H2B-GFP-Positive Neurons

The current data suggest that H2B-GFP-positive neurons are reactivated during testing when animals retrieve a memory for the training context. However, it is possible that reactivation is driven by other stimuli that are present during training and testing (e.g., transport cues, experimenter, and removal from the homecage). To examine this issue, we quantified double labeling in mice that were trained in context A and tested in a different environment (context B) 2 days later. These animals were exposed to the same background cues during training and testing but should not retrieve a memory for context A. Consistent with this idea, freezing levels in context B $(\bar{x} = 5\%)$ were significantly lower than those observed in

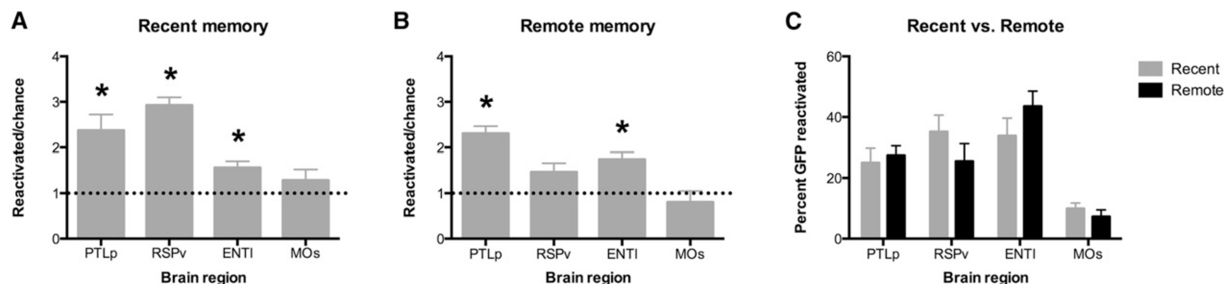

Figure 4. Reactivation of Neurons in the Cortex during Memory Retrieval

(A) TetTag mice were trained off DOX in context A and tested in the same environment 2 days later (n = 5). The percentage of double-labeled neurons is shown relative to chance (percent H2B-GFP × percent c-*fos*). Significant reactivation was observed in ENTl, RSPv, and PTLp. Reactivation was not observed in MOs.

(B) Two weeks after learning, reactivation exceeded chance in ENTl and PTLp but not RSPv or MOs.

(C) The percentage of H2B-GFP-positive neurons that were reactivated (i.e., colabeled with c-*fos*) is shown for mice tested 2 days (recent, n = 5) or 14 days (remote, n = 4) after training. The percentage of reactivated neurons did not change over time in any of the cortical regions examined. Error bars represent ± SEM. *p < 0.05.

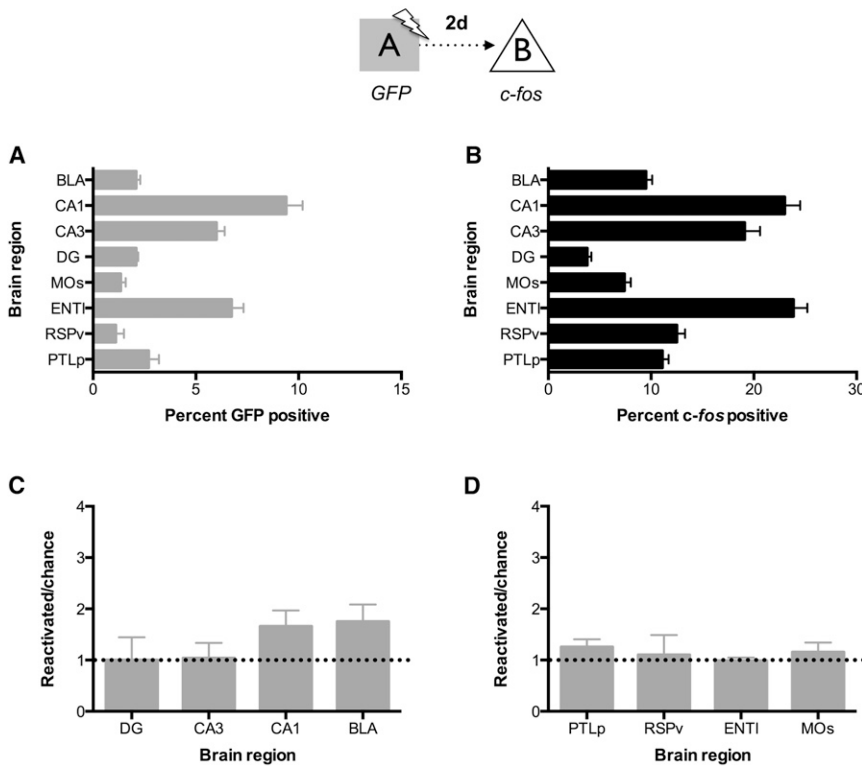

Figure 5. Memory Retrieval Is Required for Reactivation

Mice (n = 5) were trained off DOX in context A and tested in context B 2 days later.

(A) The percentage of neurons expressing H2B-GFP across brain regions.

(B) The percentage of neurons expressing c-*fos* across brain regions.

(C) The percentage of double-labeled neurons (H2B-GFP + c-*fos*) is shown relative to chance (percent H2B-GFP × percent c-*fos*). Mice trained in context A and tested in context B did not show greater than chance reactivation in the hippocampus or BLA.

(D) Mice trained and tested in context B did not show greater than chance reactivation in any of the cortical regions examined. Error bars represent ± SEM. *p < 0.05.

our previous experiment, when mice were trained and tested in context A ($\bar{x} = 30\%$) (main effect of group F (1, 8) = 5.85, $p < 0.05$). Figures 5A and 5B show the percentage of neurons expressing H2B-GFP and c-*fos* in the hippocampus, amygdala, and cortex. Figures 5C and 5D illustrate the percentage of reactivated neurons relative to chance (chance = percent GFP × percent c-*fos*). Analysis of double labeling for H2B-GFP and c-*fos* revealed that reactivation did not exceed chance in any of the brain regions examined (Fisher's PLSD, pairwise comparisons, all p values > 0.05). These data suggest that reactivation of neural networks is only observed when memory is retrieved. However, there was a trend for reactivation in the BLA and CA1 suggesting that neurons in these regions may be sensitive to other stimuli that are present during training and testing. Nonetheless, our experiments demonstrate that the main determinant of reactivation is exposure to the context in which footshock was previously administered.

Discussion

The current data demonstrate that memory retrieval involves widespread reactivation of neural ensembles that were engaged during learning. Using H2B-GFP TetTag mice, we found significant reactivation in several regions of the hippocampus, amygdala, and cortex during the retrieval of a recently formed context fear memory. Reactivation was not observed when mice were trained and tested in different environments. Similar results have been obtained in FISH studies in which rats explored the same spatial environment twice within a 30 min period [8, 9]. Our experiments extend these findings to context fear conditioning using memory tests that were conducted days and weeks after learning. Our results are also unique in that reactivation was observed even though behavioral responses were distinct during training and testing (exploration versus freezing). This implies that neural ensembles activated during exploration can be reactivated during subsequent memory retrieval when animals are immobile. To illustrate this point, Figure 6A shows the amount of activity observed during training and testing in context A (group AA, Recent). Mice were significantly more active during the training session compared to the testing session (main effect of session (1, 4) = 72.43, $p < 0.05$). The only reduction in activity that was observed during training occurred late in the session after footshocks were presented (bins 10–12). Figure 6B shows the amount of freezing observed during the same sessions. As expected, mice froze substantially more during the testing session compared to the training session (main effect of session (1, 4) = 8.29, $p < 0.05$).

It is possible that memory retrieval is not required for reactivation of H2B-GFP-positive neurons (e.g., exposure to the same sensory cues on two different occasions may simply activate a similar population of neurons). However, place cell work has found that repeated exploration of a spatial environment does not reactivate the same neurons unless plasticity mechanisms are engaged during learning. For example, if NMDARs are blocked during exploration of a novel context, place fields form but they are not stable [38]. When the animal is subsequently returned to the same context, new place fields are observed as if the rat is in a different environment. Therefore, exploring the same physical environment is not sufficient to reactivate the same group of neurons. Instead, learning needs to take place during initial exploration so that the same spatial representation can be reactivated when the animals are returned to the environment. A recent paper found that blockade of PKMζ, which impairs memory retrieval, also results in place cell remapping in a familiar environment [39]. These results suggest that reactivation of neurons in the hippocampus is contingent on memory formation and retrieval.

Another piece of evidence comes from a recent study that induced memory retrieval by selectively stimulating neurons that were engaged during learning [19]. In these experiments, a fos-tTA mouse was used to drive expression of channelrhodopsin (ChR2) in the DG during context fear conditioning.

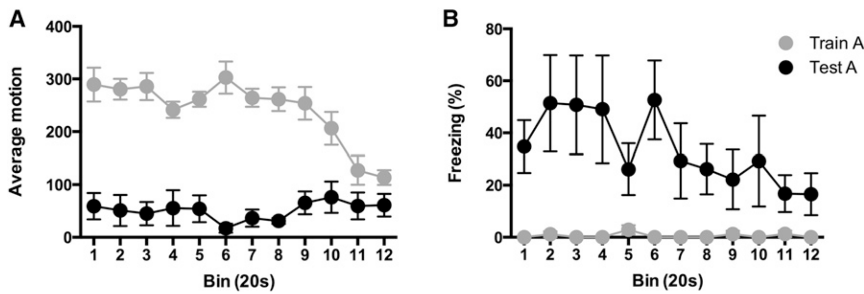

Figure 6. Exploration Differences during Training and Testing

(A) Average motion scores for mice trained and tested in context A. Mice were significantly more active during the training session compared to the testing session 2 days later. Activity only decreased during training after shock was presented (bins 10–12).

(B) Freezing data for the same training and testing sessions. There was significantly more freezing during the testing session compared to the training session. Error bars represent ± SEM.

Subsequent stimulation of c-*fos*-positive neurons resulted in memory retrieval (i.e., freezing). This result is direct evidence that reactivated neurons play a role in memory retrieval.

Within a few weeks of learning, we observed that the degree of reactivation changed in several brain regions. In the hippocampus, the percentage of reactivated neurons decreased in the DG, increased in CA3, and remained stable in CA1. The continuous generation of new neurons in the DG may contribute to the loss of reactivation in this region. Recent data indicate that neurogenesis in the DG plays an essential role in the clearance of previously formed context fear memories [40, 41]. Based on these data, one would predict a gradual decline in the reactivation of DG neurons after learning. As the percentage of reactivated neurons decreased in the DG, we observed a corresponding increase in CA3. This finding may be related to the role that CA3 plays in pattern completion. Several studies have shown that context memories lose details and become less precise with the passage of time [42, 43]. This implies that remote memory retrieval requires reactivation of partially degraded information, a process that is known to depend on CA3 [44, 45]. Therefore, as reactivation of DG neurons decreases over time, memory retrieval may be supported by reactivation of CA3 and CA1 networks.

Neurons in the CA1 region of the hippocampus were reactivated during the retrieval of recent and remote context fear memories. This result was unexpected given that the hippocampus was not required for memory retrieval 2 weeks after learning (Figure 3A). If memory can be retrieved without the hippocampus at this test interval, then why is CA1 reactivated? One possibility is that remote memory retrieval normally involves the hippocampus but can be mediated by other structures if this region is compromised. Two recent studies support this idea. The first showed that, under some conditions, inactivation of the hippocampus does not impair context fear, although it significantly alters the quality of memory that can be retrieved [30]. This suggests that alternative brain regions can retrieve information that supports freezing if the hippocampus is compromised. The second study found that prolonged inhibition of the dorsal hippocampus produces compensatory changes in the anterior cingulate cortex (ACC) that were sufficient to support memory retrieval [34]. Together, these data indicate that the hippocampus (1) is required for recent memory retrieval and (2) contributes to, but is not essential for, remote memory retrieval.

We also observed robust reactivation of cortical regions involved in spatial and contextual learning (ENTl, RSPv, and PTLp) [24, 26–28]. Reactivation of H2B-GFP-positive neurons in these regions was similar during the retrieval of recent and remote context fear memories (Figure 4C). These results are consistent with models of memory consolidation that predict stable reactivation of cortical neurons over time. According to these models, consolidation involves a gradual strengthening of intracortical connections between neurons that were coactive during learning [3, 46]. Shortly after learning, reactivation of these networks is assumed to require input from the hippocampus. However, once intracortical connections have been strengthened, cortical networks are thought to reactivate without the hippocampus. Consequently, cortical neurons should be reactivated during the retrieval of new and old memories as was observed in the current experiments.

In contrast to the cortex, there is disagreement about whether the hippocampus should be reactivated during the retrieval of remote memories. Some models predict a gradual loss of hippocampal memory traces during consolidation, while others argue that information is permanently stored in this structure [3, 47–49]. Our data indicate that some regions of the hippocampus continue to be reactivated during retrieval even when cortical regions are capable of supporting memory. This result is consistent with the idea that hippocampal representations are maintained over time as complementary traces are established outside this structure [35, 48]. Our data also suggest that while cortical traces are being established, the hippocampal representation undergoes a qualitative change that results in decreased involvement of the DG and increased involvement of the CA3 region.

Experimental Procedures

Subjects

The TetTag mice used in these experiments have been described previously [10]. These animals were generated by crossing transgenic mice that express a histone 2B-GFP fusion protein controlled by the tetO promoter (strain Tg(tetO-HIST1H2BJ/GFP) 47Efu/J; stock number 005104; Jackson Laboratory) with mice that express tetracycline-transactivator (tTA) protein under control of the c-*fos* promoter (fos-tTA; provided by Mark Mayford). TetTag mice were maintained in a C57BL/6J background. B6/129 F1 hybrids were generated by breeding TetTag mice with 129S6 mice (Taconic). Mice were born and raised on DOX chow (40 mg/kg) to prevent H2B-GFP expression prior to experimental manipulations. Mice remained on a 12 hr light/dark cycle with ad libitum access to food and water for the duration of the experiment. Experimental manipulations were performed on 8- to 12-week-old male and female mice during the light phase of the cycle. All experiments were approved by the University of Virginia Animal Care and Use Committee.

Apparatus

The contextual fear conditioning equipment used in these experiments was described previously [10]. Briefly, mice were trained in conditioning chambers (context A) that were housed in sound-attenuated boxes. The chambers contained a stainless steel grid floor, overhead LED lighting (providing broad spectrum and infrared light), and a scanning charge-coupled device video camera (Med Associates). The chamber and drop pan were cleaned with 95% ethanol before each training session. Contextual fear memory was assessed by placing the mice in context A or a new environment (context B) and measuring the freezing response. Freezing measurements were automated using the VideoFreeze system

(Med Associates) [33]. In context B, all visible light was turned off, a curved white plastic back wall was inserted, a white plastic insert covered the floor, and Saniwipes (Nice-Pak Products) were used to clean the chamber.

Behavioral Procedures

In the first experiment (Figure 1), TetTag mice in a C57BL/6J background were taken off DOX 5 days prior to training. During conditioning, mice were allowed to explore context A for 3 min prior to the onset of five footshocks (0.75 mA, 2 s) separated by a 20 s intertrial interval (ITI). In all subsequent experiments, TetTag mice on a B6/129 F1 hybrid background were taken off DOX for 3 days and trained with three shocks (0.5 mA, 2 s) to achieve a similar level of freezing and H2B-GFP expression as observed in C57BL/6J mice. Unsignaled shocks were used because a previous experiment from our laboratory showed that similar training parameters produced temporally graded retrograde amnesia [30]. After training, all mice were given high concentration DOX chow (1 g/kg) for 24 hr to rapidly suppress GFP tagging and then remained on 40 mg/kg DOX chow for the duration of the study. Memory was assessed 2 days or 14 days later by returning the mice to the training context (A) or a novel environment (B) for 5 min and measuring the freezing response.

Immunohistochemistry

Ninety minutes after behavioral testing, mice were transcardially perfused with 4% PFA, followed by 24 hr postfixation in the same solution. Free-floating 40 μm coronal sections were prepared using a vibrotome and stained in a solution containing 0.1% BSA, 0.2% Triton X, 2% normal goat serum, and anti-c-*fos* rabbit primary antibody (Calbiochem), (1:20,000 dilution for 48 hr at 4°C). After a series of 0.1 M phosphate buffer washes, sections were stained using the same blocking solution as above and Dylight 649 goat anti-rabbit secondary antibody (Jackson Immuno Research) (1:500 dilution for 24 hr at 4°C). Finally, sections were stained with DAPI (Invitrogen) (1:1,000 dilution for 5 min) and mounted on slides. In some sections, excitatory neurons were identified using an anti-CaM Kinase II, α subunit, clone 6G9 mouse primary antibody (Millipore) (1:1,000 dilution for 48 hr at 4°C), followed by anti-mouse IgG (Fab Specific)-peroxidase secondary antibody made in goat (Sigma Aldrich) (1:1,000 dilution for 24 hr at 4°C). In these sections, staining was amplified using a TSA Cyanine 5 tyramide reagent amplification system (Perkin-Elmer) (1:50 dilution).

Fluorescent Microscopy

Sections from −2.055 mm to −2.25 mm posterior to bregma were used in all experiments. Within these coordinates, we selected regions of interest in the dorsal hippocampus (DG, CA3, CA1), layer II/III of the lateral entorhinal cortex (ENTl), the basolateral nucleus of the amygdala (BLA), layer V/VI of the posterior parietal association area (PTLp), and layer II/III of the retrosplenial cortex (RSPv) based on previous studies (Figure 2) [9, 10, 14, 26]. Sections from −0.88 mm to −1.655 mm posterior to bregma were used in the analysis of a control brain region, the secondary motor cortex (MOs). Images were taken in the z plane at a frequency of 1–2 μm and acquired using 40× (DG, CA3, CA1, ENTl) or 20× (BLA, PTLp, RSPv, MOs) objective lenses of a Nikon Eclipse 80i epiflourescence microscope and NIS Elements software (Nikon). The Allen Brain Atlas (Allen Brain Atlas Resources) and a dark-field filter were used to identify each structure.

Signal Quantification

H2B-GFP and αCAMKII colocalization was determined by examining the overlap between pixels in the FITC (GFP) and CY5 (αCAMKII staining) channels. Colocalization occurred when GFP staining in the nucleus was surrounded by cytosolic staining for αCAMKII. For quantification, the middle step of each image stack was used to determine the number of DAPI-labeled nuclei expressing c-*fos* and/or GFP. To aid in quantification, we wrote a Nikon Elements macro that highlighted potential signals in the c-*fos* and GFP channels as regions of interest (ROIs) based on signal intensity ($\geq$2 SD above mean intensity of the image channel), size ($\leq \mu m^2$ area of a single DAPI labeled nuclei), and circularity. Signal quality and z stack position were then examined by a blinded rater before cells were counted as positive. To determine colocalization, we merged the c-*fos* ROI-highlighted channel with the GFP channel, allowing a rater to determine whether both signals were present in the same cell body. Cells were determined to be c-*fos* positive, GFP positive, GFP+c-*fos* positive, or signal negative. The total number of DAPI cell bodies in each step was counted manually. Bilateral DG, CA1, CA3, ENTl, BLA, PTLp, RSPv, and MOs images were quantified from five sections per animal, giving a total of ten images per region per animal. The percentage of DAPI-labeled cells containing c-*fos*, GFP, or both was calculated for each image and then averaged to produce a single measurement in each region for each animal.

Hippocampus Inactivation

Seven days prior to testing in context A, mice underwent surgery to implant plastic guide cannulae (22G; Plastics One) bilaterally into the dorsal hippocampus. Surgical procedures were similar to those described previously [10]. Briefly, mice were anesthetized with isoflurane and mounted in a stereotaxic apparatus (David Kopf Instruments). Bregma and lambda were placed in the same horizontal plane. Cannulae were inserted through small burr holes and affixed with dental cement (Harry J. Bosworth) at the following positions relative to bregma: AP, −2 mm; ML, ±1.5 mm; DV, −1 mm (from skull). Prior to testing in context A, mice were lightly anesthetized with isoflurane and injection cannulae (28G) projecting 1 mm from the tip of the guide cannulae were used to infuse the AMPA/Kainate antagonist CNQX (Sigma Aldrich) (3 mM) or saline (0.9%) into the dorsal hippocampus (0.5 μl/side; 0.1 μl/min). Injectors remained in place for 2 min to allow for diffusion. Mice were tested in context A 30 min after the infusion. Cannulae placement was confirmed after testing using the Allen Reference Atlas [50].

Statistics

Group differences were analyzed using one-way ANOVAs and planned comparisons (Fisher's PLSD). In some experiments, the percentage of double-labeled neurons $((GFP + c-fos/DAPI) \times 100)$ was compared to chance $((GFP/DAPI \times c-fos/DAPI) \times 100)$. Statistical significance for all tests was set at $p < 0.05$.

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