Irreplaceability of Neuronal Ensembles after Memory Allocation.

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Graphical Abstract

Highlights

- Inhibiting learning-activated neural ensembles impairs memory retrieval
- Inhibiting neural ensembles activated during learning impairs relearning
- Inhibiting neural ensembles for a specific memory does not hinder distinct learning

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In Brief

Is the same ensemble of neurons always dedicated to the same learning? Matsuo shows that suppression of neuronal ensembles activated during initial learning hinders relearning. This ensemble inflexibility could ensure the strengthening of synaptic connections across a specific subset of neurons by repetitive activation, thereby enabling memory enhancement.
Irreplaceability of Neuronal Ensembles after Memory Allocation

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SUMMARY

Lesion studies suggest that an alternative system can compensate for damage to the primary region employed when animals acquire a memory. However, it is unclear whether functional compensation occurs at the cellular ensemble level. Here, we inhibited the activities of a specific subset of neurons activated during initial learning by utilizing a transgenic mouse that expresses tetanus toxin (TeNT) under the control of the c-fos promoter. Notably, suppression interfered with relearning while sparing the ability to acquire and express fear memory for a distinct context. These results suggest that the activity of the initial ensemble is preferentially dedicated to the same learning and that it is not replaceable once it is allocated. Our results provide substantial insights into the machinery underlying how the brain allocates individual memories to discrete neuronal ensembles and how it ensures that repetitive learning strengthens memory by reactivating the same neuronal ensembles.

INTRODUCTION

External information acquired through daily experiences can be internally represented and stored in the brain across several interacting regions as a memory. Recent innovative studies have begun to present direct evidence that individual memories reside in the activities of specific spatially distributed neuronal populations within neuronal networks. For instance, activity manipulation of a small, specific, dispersed subset of neurons that was activated during a learning paradigm enabled memory operations including artificial retrieval and association of a previously obtained memory in mice (Garner et al., 2012; Liu et al., 2012; Ramirez et al., 2013). These studies provided a causal sufficiency between memory engrams and the activities of specific subsets of neurons.

The next critical question arising from this idea is how specific subsets of neurons are chosen from a large population of neurons to encode a given memory (Silva et al., 2009). Findings from recent research suggested a potential mechanism that involves neuronal competition. A subset of lateral amygdala neurons, in which cyclic AMP responsive element binding protein (CREB) was virally overexpressed, preferentially participated in auditory fear memory formation (Han et al., 2007). Moreover, the higher levels of CREB expression have been suggested to increase the intrinsic excitability of the neuron (Zhou et al., 2009). Thus, it is likely that neurons that are more excitable than their neighbors tend to be recruited for encoding a new memory (Yiu et al., 2014). However, much remains to be elucidated concerning the machinery of memory allocation. One of the most interesting questions is whether the same ensemble of neurons is always dedicated to the same learning or whether an alternate ensemble is flexibly substitutable. This question is critical, because it might explain how repeated training strengthens the memory. It has been well recognized that established memories can be strengthened by repeated rehearsal learning (Ebbinghaus, 1913). This is assumed to be based on the principle that the same neurons and synapses are engaged in the same learning, thereby enhancing the particular plasticity. However, it has not been demonstrated experimentally that such a mechanism actually exists in the brain. To address this particular question, a pinpoint approach is required to manipulate a specific neuronal population that is sparsely distributed in the tissue while leaving their intermingled neighbors intact. However, classical lesion or pharmacological approaches are not technically feasible.

To circumvent this difficulty, we used the c-fos-promoter-driven tTA (tetracycline-controlled transactivator) transgenic (Tg) system in mice to manipulate specific subsets of neurons in which the promoter of the c-fos gene, an immediate early gene, was activated during a given time window (Matsuo et al., 2008; Reijmers et al., 2007). In the present study, we set out to examine the impact of silencing the neuronal ensembles activated during fear-conditioned learning on memory recall, and we then tested whether the silencing interfered with subsequent relearning in the Tg mice.

RESULTS

The Transgenic System for Reversible Suppression of a Behaviorally Activated Ensemble of Neurons

The neuronal activity-dependent c-fos-promoter-driven tTA Tg system permits tagging of specific subsets of neurons that are activated during a behavioral paradigm within a given time.
window (Matsuo et al., 2008; Reijmers et al., 2007). In combination with this system, we utilized a tetanus toxin light chain (TeNT) to selectively suppress the synaptic transmission of tagged neurons and their relevant neural networks. TeNT selectively cleaves VAMP2 (also known as synaptobrevin), a synaptic vesicle protein essential for exocytosis, thereby blocking neurotransmitter release from presynaptic terminals (Link et al., 1992; Schiavo et al., 1992). By crossing the c-fos-tTA and tetO-EGFP TeNT (Yamamoto et al., 2003) Tg mice, we generated double-Tg mice (described as TeNT c-fos Tg mice) that expressed the EGFP-TeNT protein under the regulation of the c-fos promoter in a doxycycline (Dox)-dependent manner (Figure 1A). In this system, when neuronal activity sufficient to activate the c-fos promoter occurs in the absence of Dox, the tTA transgene is expressed and drives the expression of the tetO-promoter-linked EGFP-TeNT selectively in those neurons activated by the behaviorally relevant events.

In situ hybridization analysis using a TeNT cRNA probe detected sparse signals selectively in the dentate gyrus of the hippocampus and the lateral and basolateral amygdala, along with very sparse signals in the hippocampal CA1, CA3 and neocortex in fear-conditioned animals (Figure S1). Immunohistochemical analysis revealed sparsely distributed somatic GFP-positive cells, most of which were co-labeled with the neuron-specific marker NeuN and the excitatory neuron marker CaMKIIα (Figures 1B–1E). The GFP expression seen in some of the cell nuclei is derived from a Fos-GFP transgene that is expressed independently of the Dox-regulated system (Matsuo et al., 2008).

In the CA1, CA3 areas and dentate gyrus of the hippocampus, larger numbers of cells expressing EGFP-TeNT were detected in fear-conditioned animals relative to home-cage controls and Dox-treated animals (Figure S2), confirming successful transgene suppression by Dox treatment and activity-dependent expression of TeNT in the absence of Dox.

**Figure 1. Transgenic System of Activity-Dependent EGFP-TeNT Expression during a Given Time Window**

(A) Schematic representation of the transgenic (Tg) system. The synthesis of EGFP-TeNT is regulated by neuronal activity via the c-fos promoter and is also dependent on the tetracycline-inducible expression system.

(B and D) Representative confocal fluorescent images showing the EGFP-TeNT expression (white arrow) in the hippocampal dentate gyrus of the TeNT c-fos Tg mouse, shown by immunohistochemistry with an anti-GFP antibody (green), anti-NeuN or CaMKIIα antibodies (red), and DAPI (blue). Scale bars, 20 μm.

(C) Percentage of NeuN+ cells within somatic GFP+ cells (96.19 ± 1.91%; three mice, n = 44 cells).

(E) Percentage of CaMKIIα+ cells within somatic GFP+ cells (96.66 ± 1.68%; three mice, n = 49 cells).

**Necessity of Learning-Activated Neuronal Ensembles for Memory Retrieval**

We first examined whether the TeNT expression that was induced during learning affected the subsequent memory expression. The TeNT c-fos Tg mice were removed from Dox for 3 days and were fear conditioned to elicit a long-term fear memory and to initiate the synthesis of TeNT selectively in the activated neurons. Mice were returned to their home cages and treated with Dox to suppress further induction of TeNT in neurons that were unrelated to the learning. On the following day, mice were re-exposed to the same chamber without footshocks to evaluate their contextual fear memory by measuring a freezing behavior (Figure 2A). The Tg mice showed significantly less freezing compared with wild-type (WT) littermates (Figure 2B; p = 0.0018, unpaired t test), suggesting an impairment of contextual fear memory retrieval in the Tg mice.
Low levels of TeNT expression induced by home-cage activities during the off-Dox period could potentially impair the memory acquisition or neurological performance. To confirm that the Tg mice successfully acquired the contextual fear memory, we performed a retrieval test 5 min after conditioning (Figure 2C). The Tg mice froze significantly ($p < 0.0001$, paired t test), and the proportion of Tg mice that froze was comparable to that of WT animals ($p = 0.6087$, unpaired t test).

The impairment of contextual fear retrieval tested 24 hr after conditioning could reflect an inhibition in a process of either memory retrieval or consolidation. To discriminate these processes, we performed a memory retrieval test 28 days after training, when TeNT expression was reversibly suppressed (Figure 2). Both WT and Tg mice exhibited robust freezing, and there was no marked difference in the retrieval performance between them (Figure 2D; $p = 0.5698$, unpaired t test), suggesting that the deficit at the retrieval test 24 hr after conditioning was caused by an impairment in retrieval rather than in consolidation. It is notable that blocking the synaptic activity of specific neuronal ensembles after learning did not impair the long-term consolidation of contextual fear memory.

We next examined the impact of the TeNT-mediated silencing on conditioned-cue memory (Figure 2E). The Tg mice significantly froze during tone presentation in a novel environment (context C) 24 hr after conditioning, at levels comparable to WT controls ($p = 0.8546$, unpaired t test). The presence of normal cued fear expression in the Tg mice indicates that the observed TeNT-mediated impairment of contextual fear is not due to deficits in pain sensitivity, motivation, fear expression system, or other general neurological functions. Taken together, our results demonstrate that the TeNT-mediated suppression of neuronal ensembles that are naturally activated during fear conditioning results in a failure of the contextual fear memory retrieval. It further indicates that the reactivation of the ensemble engaged in memory acquisition is necessary for the retrieval.

**Necessity of Neuronal Ensembles Activated during Initial Learning for Relearning**

Next, we investigated whether inhibiting the reactivation of the neuronal ensembles that participated in the initial learning could hinder relearning. If the same subset of neurons is not necessarily assigned to the same learning and an alternative ensemble of neurons can functionally compensate for the inhibited ensemble, animals should acquire and express the fear memory. To test this idea, TeNT-c-fos mice were fear conditioned in context A in the absence of Dox to induce TeNT expression in the activated neurons. Then, they were retrained in the same chamber (context A) 24 hr after the initial training in the presence of Dox. Animals were subsequently re-exposed to the same environment to assess the contextual fear memory elicited by the second conditioning trial (Figure 3A). Notably, freezing was not increased compared with pre-retraining duration (Figure 3B; two-way ANOVA, $p = 0.5671$), indicating that the second training session failed to strengthen the contextual fear memory in these mice. In contrast, WT control animals exhibited substantially increased freezing after retraining (Figure 3B; two-way ANOVA, $p = 0.0045$; see Figure 3B legend), demonstrating that the memory was strengthened by the same additional training in context A. This also indicates that the failure of enhanced freezing in the Tg mice was not due to a ceiling effect.

To examine the specificity of the TeNT-mediated silencing to discrete neuronal representations, we investigated whether the Tg mice were able to acquire a new fear memory associated with a different context. Animals were fear conditioned in context A in the absence of Dox and retrained in a distinct chamber (context B) 24 hr later in the presence of Dox (Figure 3A). We found that they showed significantly more freezing during the retrieval test than during the period before shock presentation at the retraining session in context B (Figure 3C; $p = 0.0011$, paired t test). The enhanced freezing level was significantly higher in mice that were retrained in context B than in mice
context A (black bar) and context B (gray bar), showing a specific inhibition of freezing (over 20%) during the first 3 min of retraining. *p < 0.05 (Mann-Whitney test). Over, we compared the freezing level after training in either context A or context B using naive WT mice to eliminate the possibility that the training in context B might elicit a stronger freezing response than training in context A (Figure S3). Instead, conditioning in context B evoked a mild freezing, with no significant difference between the contexts (p = 0.2105, unpaired t test).

In summary, our data suggest that TeNT expression in neurons activated during memory encoding selectively inhibits relearning without disrupting the ability to acquire and retrieve a fear memory for distinct context. These results indicate that there is a mechanism ensuring that the same neuronal ensemble is engaged for the same learning to strengthen the memory and that it is not substitutable after the ensemble is allocated for the initial learning.

**DISCUSSION**

In the present study, we have generated a double-Tg mouse in which a synaptic transmission of neuronal ensembles activated by a given behavioral stimulus was selectively and reversibly inhibited. Using this Tg mouse, we have demonstrated the following: (1) suppression of neuronal ensembles that were naturally activated during fear-conditioned learning impaired the retrieval of the contextual fear memory, and (2) suppression of neuronal ensembles that were activated during fear-conditioned learning hindered relearning of the memory but did not interfere with new learning of a distinct contextual fear memory.

Identifying a neuronal circuit or population of neurons responsible for a particular behavior is one of the major challenges in neuroscience. Previous cellular imaging studies have found positive correlations between the activity of a small subset of neurons activated during learning and the performance of recall in several brain regions, such as the amygdala and hippocampus (Reijmers et al., 2007; Tayler et al., 2013). Thus, memory retrieval is assumed to involve the reactivation of neural ensembles that were established during encoding. To show the causal necessity, a series of experiments has elegantly shown that selective ablation or inactivation of a subpopulation of cells in the lateral amygdala that overexpressed CREB resulted in a disruption of the fear memory (Han et al., 2009; Zhou et al., 2009). However, these studies did not directly address whether the reactivation of the neuronal population that is naturally activated during learning is required for memory recall, because cells were artificially forced to allocate to the component constituting an associative memory. A genetic tagging system based on the neuronal activity-dependent c-fos gene promoter (Matsu et al., 2008; Reijmers et al., 2007) enabled us to examine the impact of inhibition of a neuronal ensemble naturally induced by fear-conditioned learning on the retrieval of that memory and to demonstrate that the reactivation of that ensemble is required for recall of the contextual fear memory. This is consistent with the recently published results using Arc promoter and optogenetics (Denny et al., 2014).

The reason of intact cued fear memory is unclear. A previous report demonstrated rats with approximately one-third of their lateral amygdala neurons infected with the plasticity-block vector showed diminished cued fear learning, whereas animals with less than 10% of neurons infected showed no effects on learning (Rumpel et al., 2005). This suggests that the expression of
induced TeNT in the amygdala was possibly too small to block the memory engram of cued fear. Interestingly, CNS-specific c-fos-knockout mice also showed a deficiency in contextual, but not cued, fear memory (Fleischmann et al., 2003). Cells tagged by c-fos-promoter activation in the amygdala might therefore not necessarily be required for cued fear memory expression.

The c-fos-promoter-induced expression of the transgene in the hippocampal CA1 and CA3 regions of the TeNTc-fos Tg mice was much smaller than that described in previous reports (Matsuo et al., 2008; Garner et al., 2012; Ramirez et al., 2013), possibly due to differences in the reporter gene expression system. In spite of the low expression of TeNT in these regions, retrieval and relearning of a contextual fear memory was impaired, implying that an extremely small population of cells might represent a particular memory. Alternatively, it is possible that inhibiting the memory engram in the dentate gyrus was sufficient to suppress contextual fear memory, supporting recent studies demonstrating causality between c-fos-activated cells in the dentate gyrus and memory engrams (Liu et al., 2012; Ramirez et al., 2013).

The hippocampus is an essential structure for contextual fear learning (Anagnostaras et al., 2001; Maren, 2001). Primarily, this is based on the fact that hippocampal lesions disrupt contextual fear learning. However, in some conditions, rodents with pretraining hippocampal lesions can acquire contextual fear memory, albeit less efficiently (Maren et al., 1997; Frankland et al., 1998; Wiltgen et al., 2006). Rats with post-training hippocampal lesions also exhibited severe retrograde amnesia but showed contextual freezing after retraining (Wiltgen et al., 2006). These results imply that an alternative system can compensate for the hippocampal damage at the structural level (Zelikowsky et al., 2013). However, lesion experiments destroy entire cells and circuits within the targeted region and thus may affect all processing that requires that structure. The current study examined whether compensation occurs for memory allocation during relearning at the cellular ensemble level, although the suppression was not specific for a particular structure. Interestingly, our results revealed that mice did not relearn when the neuronal ensemble engaged in the initial learning was compromised, indicating that functional compensation did not occur. Importantly, mice were capable of acquiring new memory for a different context, demonstrating an ensemble-specific, but not an overall non-selective, effect. Therefore, in contrast to cases in which the primary responsible region was entirely damaged (Maren et al., 1997; Frankland et al., 1998; Wiltgen et al., 2006; Zelikowsky et al., 2013), alternative ensembles are not likely to substitute for the primary cellular ensembles once they are allocated for a given representation. Suppression of relearning might require that the entire associative network containing the memory be compromised, because the formation of memory representation is likely to depend on complex interplay among memory traces in the brain, including the primary sensory cortices (Gdalyahu et al., 2012; Xie et al., 2014). Alternatively, it would be interesting to test whether suppression of ensembles in a particularly restricted brain region is sufficient to inhibit relearning.

Established memories can be strengthened by repeated rehearsal learning (Ebbinghaus, 1913). However, the underlying neural mechanism remains to be elucidated. Our result provides remarkable insight, because it implies that the same neuronal ensemble is preferentially dedicated to the repetitive learning. This inflexibility of an ensemble could ensure the strengthening of synaptic connections across a specific subset of neurons by repetitive activation, thereby enabling memory enhancement.

EXPERIMENTAL PROCEDURES

Subjects

The TeNTc-fos/Tg mice were generated by crossing c-fos-TTA-Tg mice (Matsuo et al., 2008; Reijmers et al., 2007) with Tg mice expressing TeNT fused to EGFP under the control of the tetO promoter (Yamamoto et al., 2003). They were bred for more than nine generations on the C57BL/6J background and maintained as heterozygotes. Animals were housed socially (two to five animals per cage) and given food containing 50 mg/kg Doox and water ad libitum. Male Tg and WT littermate mice (11–14 weeks old) were used for behavioral experiments. All procedures were approved and conducted in accordance with the guidelines of Kyoto University and Osaka University on the care and use of laboratory animals.

Contextual and Cued Fear Conditioning

All behavioral experiments were conducted during the light period of the light/dark cycle. The mice were housed individually for at least 6 days before the onset of experiments, and they were handled for 4 days. They were placed in a novel rectangular chamber (33 × 25 × 28 cm, 100 lux) with white plastic side walls, a transparent plastic top and front and rear walls, and a stainless-steel grid floor (O’Hara) (context A) and were allowed to explore freely for 3 min. After the 3-min baseline period, three tone-shock pairings were presented. Each pairing consisted of a 30-s, 55-dB white noise ending simultaneously with a 2-s, 0.20-mA footshock. There was a 1-min interval between each pairing. The mice remained in the chamber for 30 s after the last footshock before being returned to their home cage. The total duration of training was 420 s.

For the contextual fear memory retrieval test, mice were returned to the conditioned chamber (context A) for 3 min to assess their contextual fear memory as measured by their freezing behavior at 5 min, 24 hr, or 28 days after conditioning. A different set of mice was used for each different time point to avoid the influences of prior retrieval and reconsolidation. To examine a cued fear memory, mice were placed in a novel triangular chamber made of opaque white Plexiglas (33 × 29 × 40 cm, 10–15 lux) that was located in a different soundproof room (context C) for 3 min (pre-tone), then subjected to 90 s of conditioned-stimulus tone exposure.

For the retraining experiment, a group of mice was retrained in context A 24 hr after the first training in context A. Another group of mice underwent the same training in context B, which consisted of a novel oval chamber with black and white checked patterned walls, a stainless-steel grid floor with staggered grid rods, and red light (15 lux) (see Figure 5F). They were subsequently subjected to a contextual fear memory test in either context A or context B.

Freezing was scored and analyzed automatically by a charge-coupled device (CCD) camera-based system, TimeFZ4 (O’Hara). Images were recorded from the top of each chamber using the camera (two frames per second). For the analysis of images, the gap area (pixels) between the contour of the mouse in one frame and that in the next frame was identified. If the gap area was under 20 pixels for 2 continuous seconds, mice were judged to have exhibited freezing behavior. Freezing scores are expressed as the ratio of the freezing period to the experimental period.

Immunohistochemistry

Brains were fixed with 4% paraformaldehyde (PFA) in PBS at 4°C overnight and sectioned at a thickness of 50 μm using a vibratome (Leica). Free-floating slices were permeabilized with 0.3% Triton X-100 in 5% BSA/PBS at room temperature for 30 min, then rinsed with PBS. For enzyme antibody staining, permeabilized slices were incubated with primary antibody (rabbit anti-GFP antibody, Invitrogen) at room temperature overnight. Slices were rinsed with 0.3% Triton X-100/PBS three times for 10 min and then incubated with

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secondary antibody (biotin-conjugated donkey anti-rabbit antibody, Millipore) at room temperature for 1 h. Slices were then rinsed with 0.3% Triton X-100/ PBS three times for 10 min and incubated with diluted avidin-biotinylated peroxidase complex (ABC-Elite, Vector Laboratories) at room temperature for 1 h. Then, slices were rinsed with PBS three times for 10 min, and incubated with a solution containing 0.02% 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma), 0.001% hydrogen peroxide, and 50 mM Tris-HCl (pH 7.8). Finally, slices were rinsed with PBS three times for 10 min and mounted in 50% glycerine. For the quantification of TeNT-EGFP-immunoreactive cells, images were acquired using an Axioplan 2 imaging microscope (Zeiss) equipped with an AxioCam HR CCD camera (Zeiss). Images were binarized using ImageJ (NIH), and TeNT-EGFP-positive cells were counted by an experimenter blind to the condition.

For fluorescent antibody staining, permeabilized slices were incubated with primary antibodies (rabbit anti-GFP antibody, Invitrogen; mouse anti-NeuN antibody, Millipore; mouse anti-OctMKII antibody, Millipore) at 4°C overnight. Slices were then rinsed with PBS for three times for 10 min and incubated with secondary antibodies at 4°C overnight (goat anti-rabbit Alexa 488, Invitrogen; goat anti-mouse Alexa Fluor 594, Invitrogen). After rinsing, slices were subsequently incubated with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) at room temperature for 10 min and then mounted in Slowfade Gold antifade reagent (Invitrogen). Fluorescent images were obtained using TCS SP8 confocal laser scanning microscopy with GaAsP hybrid detectors (Leica).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.03.042.

REFERENCES


