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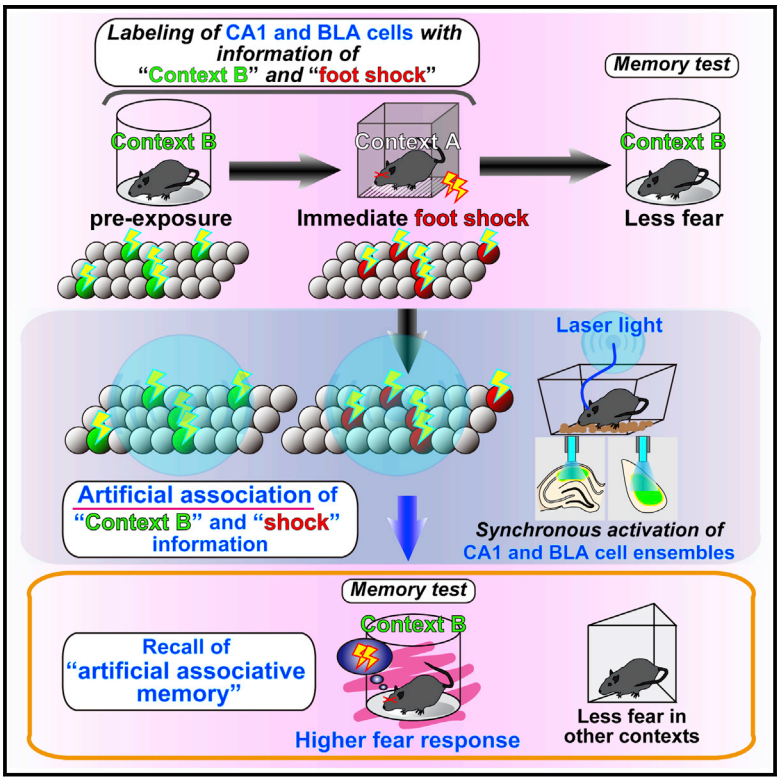
Artificial Association of Pre-stored Information to Generate a Qualitatively New Memory

Graphical Abstract



Highlights

- Context exposure and foot shock form cell ensembles in the CA1 and the BLA
- Synchronous activation of the ensembles artificially associates less-linked episodes
- The artificial association requires activation of both the CA1 and the BLA
- The artificial and physiological associations share the same molecular mechanisms

Authors

Noriaki Ohkawa, Yoshito Saitoh, ..., Fusao Kato, Kaoru Inokuchi

Correspondence

inokuchi@med.u-toyama.ac.jp

In Brief

Although memories are stored in the brain as cellular ensembles, it is unclear how distinct information becomes associated at the cell ensemble level. Ohkawa et al. find that coincident firing of distinct neuronal ensembles generates a link between these cell ensembles leading to an association of originally independent memory episodes.

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Artificial Association of Pre-stored Information to Generate a Qualitatively New Memory

Noriaki Ohkawa,[1,2] Yoshito Saitoh,[1,2] Akinobu Suzuki,[1,2] Shuhei Tsujimura,[1,2] Emi Murayama,[1,2] Sakurako Kosugi,[1,2] Hirofumi Nishizono,[3] Mina Matsuo,[3] Yukari Takahashi,[4] Masashi Nagase,[4] Yae K. Sugimura,[4] Ayako M. Watabe,[4,5] Fusao Kato,[4,5] and Kaoru Inokuchi[1,2,*]

[1]Department of Biochemistry, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
[2]CREST, JST, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
[3]Division of Animal Experimental Laboratory, Life Science Research Center, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
[4]Department of Neuroscience, Jikei University School of Medicine, Tokyo 105-8461, Japan
[5]Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan
*Correspondence: inokuchi@med.u-toyama.ac.jp
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SUMMARY

Memory is thought to be stored in the brain as an ensemble of cells activated during learning. Although optical stimulation of a cell ensemble triggers the retrieval of the corresponding memory, it is unclear how the association of information occurs at the cell ensemble level. Using optogenetic stimulation without any sensory input in mice, we found that an artificial association between stored, non-related contextual, and fear information was generated through the synchronous activation of distinct cell ensembles corresponding to the stored information. This artificial association shared characteristics with physiologically associated memories, such as N-methyl-D-aspartate receptor activity and protein synthesis dependence. These findings suggest that the association of information is achieved through the synchronous activity of distinct cell ensembles. This mechanism may underlie memory updating by incorporating novel information into pre-existing networks to form qualitatively new memories.

INTRODUCTION

It is unclear how distinct units of information become associated in the brain. Recent studies reveal that subpopulations of neurons activated during initial learning are reactivated during retrieval (Deng et al., 2013; Guzowski et al., 1999; Ramirez et al., 2013; Reijmers et al., 2007; Tayler et al., 2013) and that reactivation of the selected neuronal ensemble is required and sufficient to retrieve that memory (Han et al., 2009; Liu et al., 2012; Rogerson et al., 2014; Silva et al., 2009). These findings indicate that information relating to an experience is encoded in a specific cell ensemble that is selected and activated during a corresponding event. Neurons showing relatively higher activity during an event are preferentially recruited to join an ensemble (Han et al., 2007, 2009; Rogerson et al., 2014; Silva et al., 2009; Zhou et al., 2009).

Although our knowledge of memory acquisition and retrieval is accumulating, little is known about the mechanisms underlying the association between individual units of information within separate neuronal ensembles. This is of interest because the association of a previously formed memory with information from a present event is thought to underlie memory updating (Debiec et al., 2006; Nader and Hardt, 2009; Tronel et al., 2005) and is suggested to be involved in post-traumatic stress disorder (Dolcos, 2013). Recent studies showed that acquisition of a new memory can be modified by the simultaneous and artificial reactivation of a specific neuronal ensemble corresponding to that pre-stored memory, thereby generating false or synthetic memories (Garner et al., 2012; Ramirez et al., 2013).

Coincident activation of neurons results in a strengthening in synaptic efficacy between those neurons (Caporale and Dan, 2008; Fell and Axmacher, 2011; Hebb, 1949). These sets of neurons become wired together through enhanced synaptic efficacy. This mechanism is thought to underlie the generation of a neuronal ensemble that encodes a particular memory.

The simultaneous retrieval of two distinct units of information stored in the brain can link the original information to create an association. We hypothesized that the coincident firing of two neuronal ensembles corresponding to two distinct memory episodes that were previously stored independently in the brain generates a strong link between these two neuronal ensembles, as co-activated cells become wired together by enhanced synaptic efficacy, leading to an association between two originally independent memories. Here, we show that synchronous reactivation achieved by artificial optical stimulation of neuronal ensembles corresponding to two distinct memory episodes is capable of generating an artificial link between these memory episodes, strongly supporting our hypothesis.

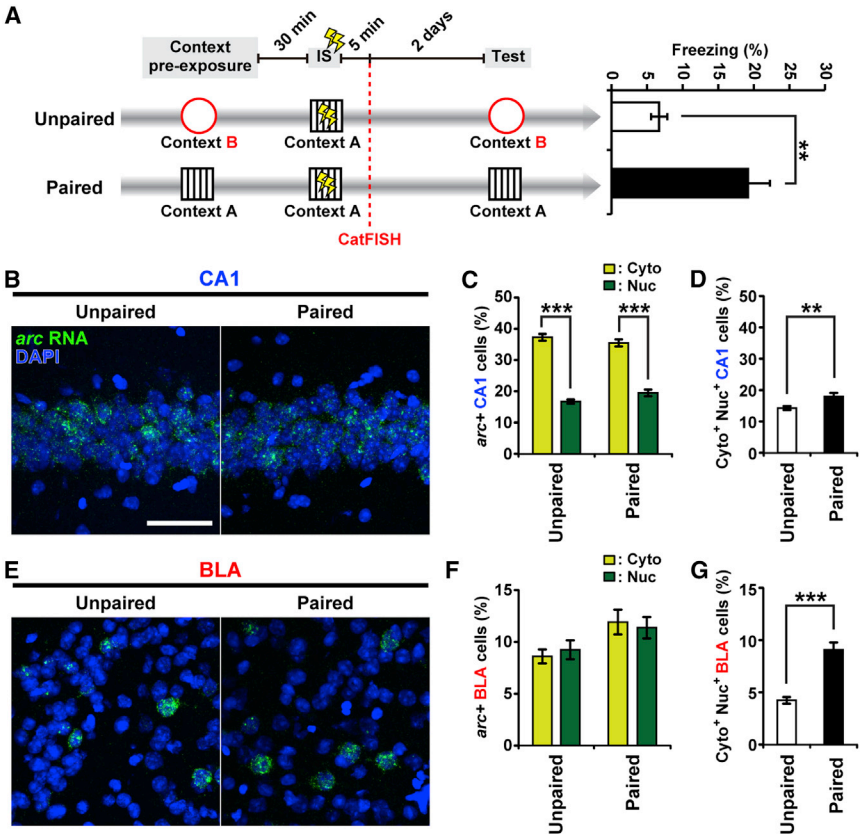


Figure 1. Cell Ensembles Corresponding to the Pre-exposed Context and the Immediate Shock in Hippocampal CA1 Region and the Basolateral Amygdala

(A) Context pre-exposure and IS paradigm. The graph shows the freezing levels during the test session in the unpaired (n = 8) and paired (n = 14) groups (**$p < 0.002$, Welch's t test, two tailed). The timing of cell compartment analysis of temporal activity using fluorescence in situ hybridization (catFISH) sampling is indicated with a red dashed line.

(B–G) Detection of cells activated during context pre-exposure and IS. (B and E) Representative z-stack images of *arc* catFISH signals captured in slices taken from the CA1 (B) and the BLA (E) of unpaired and paired mice. The *arc* RNA signal and DAPI nuclear staining are shown in green and blue, respectively. Scale bar, 50 μm. (C and F) Graphs showing the percentage of cells containing cytoplasmic *arc* (Cyto) and nuclear *arc* (Nuc) RNA in DAPI-positive cells of the CA1 (C) and BLA (F) (n = 12 sections from four mice/group). (D and G) The number of Cyto and Nuc RNA double-positive cells in the CA1 (D) and BLA (G) (n = 12 sections from four mice/group). (C) ***$p < 0.001$, unpaired Student's t test, two tailed. (D) **$p < 0.01$, unpaired Student's t test, two tailed. (G) ***$p < 0.001$, Welch's t test, two tailed. Error bars, mean ± SEM. See also Figure S1.

RESULTS

A Context Pre-exposure and Immediate Shock Paradigm Establishes Corresponding Cell Ensembles in the Hippocampus and Amygdala

We took advantage of a context pre-exposure and immediate shock (IS) paradigm suitable for analyzing the association between two experiences, i.e., the context and an electric foot shock (unconditioned stimulus). In this paradigm, mice associated context A, to which they had been previously exposed (pre-exposed context), with an IS delivered in the same context (the paired condition), resulting in an increase in freezing behavior compared with non-pre-exposed mice in a test session in context A (Figures S1A–S1C); this is consistent with the results of previous reports (Fanselow, 1990; Rudy and O'Reilly, 2001). When different contexts were used for the pre-exposure (context B) and the IS (in context A) (the unpaired condition), mice spent significantly less time freezing during the test session in context B compared with mice in the paired condition (Figure 1A).

Hippocampal and amygdala N-methyl-D-aspartate receptor (NMDAR) function during the context pre-exposure and IS, respectively, are required to form associative fear memory (Matus-Amat et al., 2007). This suggests that a major component of the cell ensemble corresponding to the pre-exposed context and the IS established in the hippocampus and amygdala. Here, we used cell compartment analysis of temporal activity using fluorescence in situ hybridization (catFISH) to detect the immediate early gene, *arc* (Guzowski et al., 1999), to determine when a subpopulation of neurons was activated during behavioral training (Figures 1B–1G and S1D; see Figure 1A for the experimental schedule). *arc* RNA localizes to the nucleus and cytoplasm 5 min and 25–35 min, respectively, after its induction (Guzowski et al., 1999). Therefore, in the present study, the expression of nuclear and cytoplasmic *arc* RNA distinguished neuronal populations engaged by the behavioral experience with the IS and the context pre-exposure, respectively. The expression of cytoplasmic or nuclear *arc*-positive cells in the CA1 and basolateral amygdala (BLA) was similar under the paired and unpaired conditions (Figures 1C and 1F). The most prominent result was a significant increase in the number of double-positive cells (i.e., *arc* signals detected in both the cytoplasm and the nucleus) in the paired condition (Figures 1D and 1G), suggesting that sharing an activated subpopulation of neurons underlies the association between the pre-exposed context and the IS experience, especially in the BLA (Matus-Amat et al., 2007).

Manipulation of Cell Ensembles in Both the Hippocampus and Amygdala

We used c-*fos*::tetracycline transactivator (::tTA) transgenic mice carrying a lentiviral vector harboring channelrhodopsin-2 (ChR2) to genetically target cell ensembles corresponding to specific events (Figure 2A). ChR2 is a light-activated cation

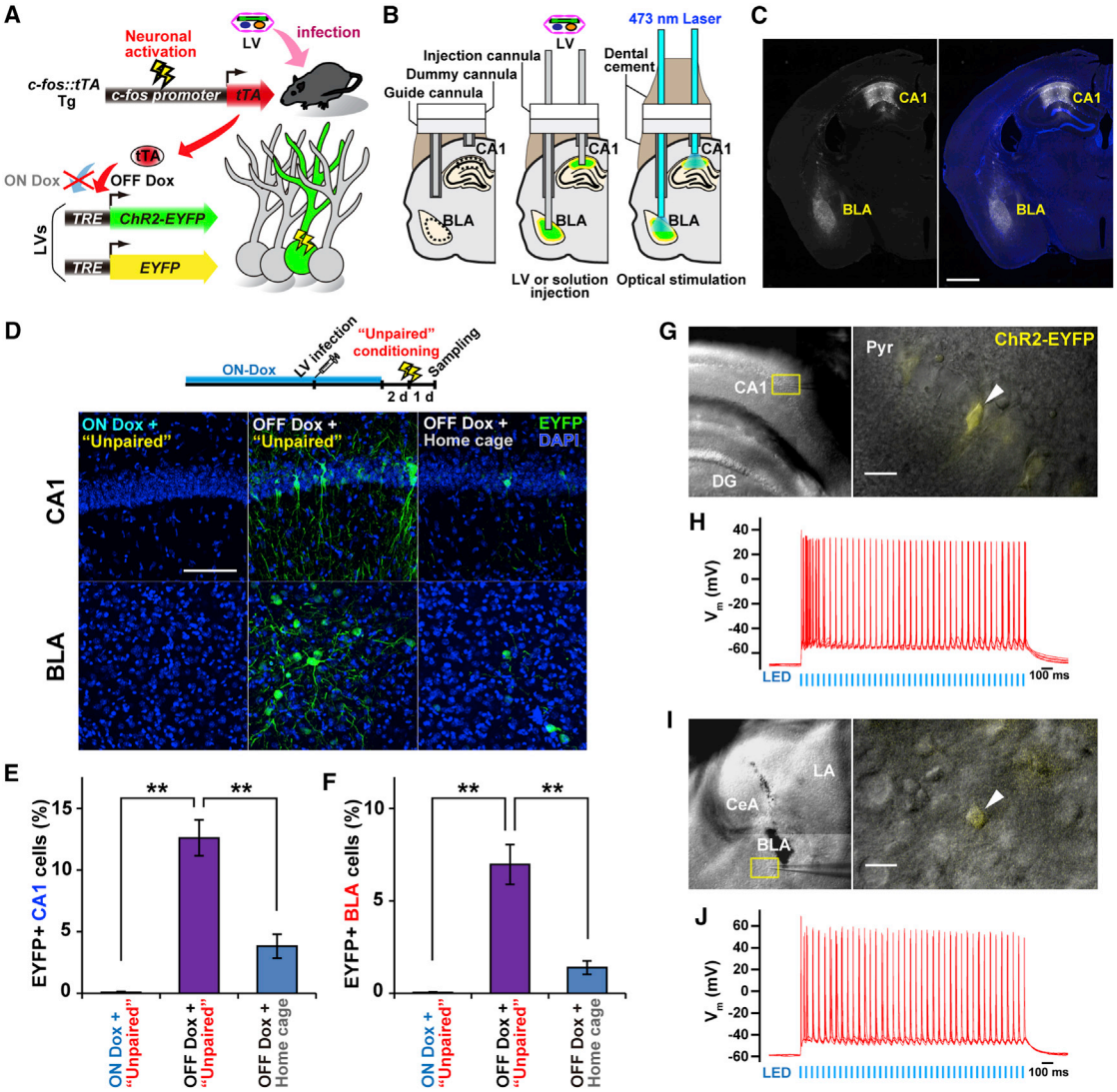


Figure 2. A System for the Simultaneous Targeting and Activation of Cell Ensembles in the Hippocampal CA1 Region and the Basolateral Amygdala

(A) Diagram showing the activity-dependent targeting of neurons in c-*fos*-tetracycline transactivator (::tTA) mice with the lentiviral (LV) system. Channelrhodopsin-2 (ChR2) and/or enhanced yellow fluorescent protein (EYFP) was selectively introduced into activated cells (green).

(B) Cannula system for double-targeting the CA1 and BLA. The system was used for both LV injection and placement of optic fibers.

(C) Representative fluorescent protein labeling patterns of CA1 and BLA cells in a c-*fos*::tTA mouse conditioned with the paired paradigm 2 days after doxycycline (Dox) removal. The fluorescent protein signal and DAPI nuclear staining 1 day after conditioning are shown in white and blue, respectively. Scale bar, 1 mm.

(D–F) The activity-dependent and OFF Dox-dependent labeling of cells with EYFP in the CA1 and BLA. (D) The upper panel shows the scheme of the experiment. Neural activity was elicited by unpaired conditioning. The thick blue line indicates the presence of Dox (ON Dox). The lower panel shows representative images of EYFP expression in the CA1 (upper) and BLA (lower) of LV-injected-c-*fos*::tTA mice subjected to the unpaired paradigm during ON Dox (left), unpaired conditioning during OFF Dox (middle), and no activity (home cage) during OFF Dox (right). The EYFP signal and DAPI nuclear staining are shown in green and blue, respectively. Scale bar, 100 μm. (E and F) Graphs showing the percentage of EYFP-positive cells to DAPI-positive cells in the CA1 (E) and BLA (F) (n = 6 sections from three mice/group). $p < 0.001$, one-way ANOVA; $^{**}p < 0.01$, Scheffe's post hoc multiple comparisons test. Error bars, mean ± SEM.

(G–J) Light-pulse-dependent spike induction in the CA1 and BLA neurons of c-*fos*::tTA mice injected with LV (TRE::ChR2-EYFP). Recorded cells in CA1 (G) and BLA (I) expressing ChR2-EYFP are indicated by arrowheads. (G and I) Magnified images are shown on the right. Yellow square, magnified area. Blue LED light was delivered (20 Hz, 5-ms pulses for 2 s), and spikes from the cells in CA1 (H) and the BLA (J) were recorded under current-clamp mode. Pyr, pyramidal cell layer; CeA, central nucleus of the amygdala; LA, lateral nucleus of amygdala. Scale bar, 20 μm.

See also Figure S2.

channel (Fenno et al., 2011). The promoter activity of the *c-fos* gene, another immediate early gene, is driven by neuronal activity. The tTA binds to a tetracycline-responsive element (TRE) and drives the expression of a target gene downstream of the TRE upon the removal of doxycycline (Dox). Therefore, in c-*fos*::tTA mice injected with a recombinant lentivirus (LV) harboring

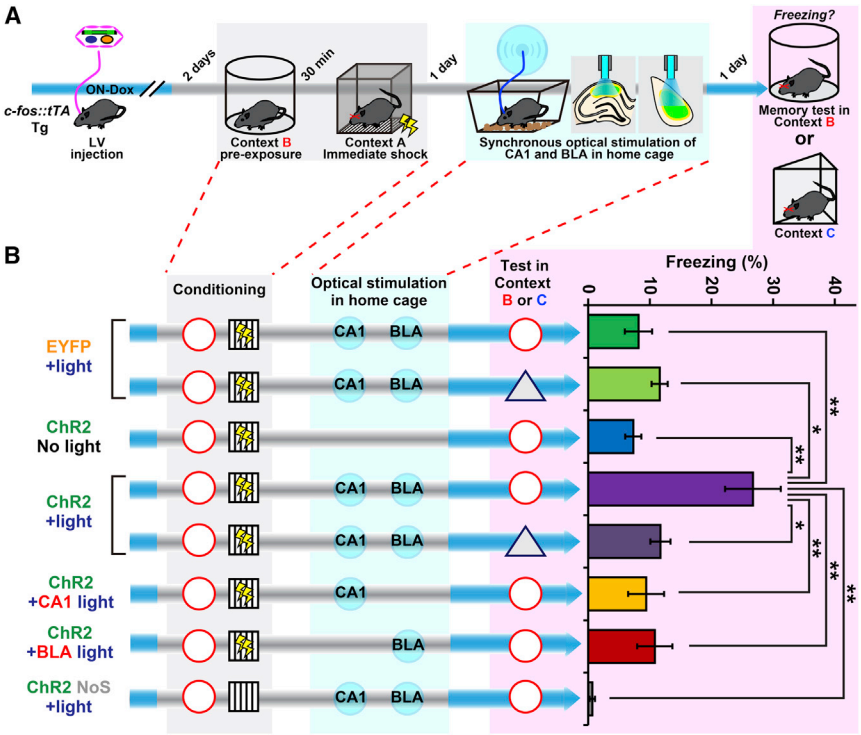


Figure 3. Optical Stimulation of Cell Ensembles in the Hippocampal CA1 and Basolateral Amygdala Generates New Associative Memory

(A) Diagram showing the experimental scheme. The thick blue and gray lines represent the presence (ON Dox) and absence (OFF Dox) of doxycycline, respectively. The blue circle represents light stimulation. The c-*fos*::tTA mice were used in all groups.

(B) All groups, except the no-shock group, were conditioned in the unpaired paradigm with OFF Dox. The enhanced yellow fluorescent protein (EYFP) + light group was injected with lentivirus (LV) (TRE::EYFP) into the CA1 and BLA, subjected to optical stimulation in the CA1 and BLA, and then tested in context B (n = 13) or C (n = 10). All channelrhodopsin-2 (ChR2) groups were injected with LV (TRE::ChR2-EYFP) into CA1 and/or the BLA, as indicated. The ChR2 no-light group received no optical stimulation and was tested in context B (n = 13). The ChR2 + light group received optical stimulation in the CA1 and BLA and was then tested in context B (n = 13) or C (n = 12). The ChR2 + CA1 light (n = 10) and the ChR2 + BLA light (n = 14) groups received optical stimulation only in the CA1 and the BLA, respectively. In the ChR2 + no shock (NoS) light group (n = 6), foot shock was not delivered during the immediate shock (IS). All other procedures were the same as those used for the ChR2 + light group. The graph shows the freezing response during the memory test ($p < 0.001$, one-way ANOVA; $^{*}p < 0.05$, $^{**}p < 0.01$, Scheffe's post hoc multiple comparisons test). Error bars, mean ± SEM.

See also Figure S3.

TRE::ChR2-enhanced yellow fluorescent protein (EYFP) or TRE::EYFP, the subpopulation of cells activated during learning were specifically labeled with ChR2 and/or fluorescent protein in the absence of Dox (OFF Dox) for 2 days.

TRE::ChR2-EYFP LV or TRE::EYFP LV was unilaterally injected into the CA1 and BLA of c-*fos*::tTA mice using a double-targeting cannula system aimed at both the CA1 and the BLA. The guide cannula was designed for use with both LV injections and optical fiber placements (Figure 2B). This system allowed us to specifically target activated cells in CA1 and the BLA (Figure 2C). Fluorescently labeled cells were predominantly observed in OFF-Dox animals, and the number of EYFP-positive cells was significantly increased by unpaired (Figures 2D–2F) and paired conditioning (Figure S2A). Following the unpaired conditioning with OFF-Dox, approximately 13% of the cells in the hippocampal CA1 and 7% of those in the BLA were labeled with EYFP (Figures 2D–2F). The labeling was not observed when unpaired conditioning was performed with ON-Dox, which does not allow tTA-dependent gene expression. Even with OFF-Dox, the labeling efficiency in mice kept in their home cages was significantly lower than that of the OFF-Dox mice that were conditioned. In addition, unpaired conditioning induced the expression of ChR2-EYFP (Figure S2B). Our results also indicated that the cells labeled with ChR2-EYFP were a subpopulation of those cells expressing *arc* RNA, because most of the *tTA* RNA-positive cells expressed *arc* RNA in the CA1 and BLA of the c-*fos*::tTA mice after the unpaired conditioning (Figure S2C).

Recordings of membrane potentials from neurons in acute hippocampal and amygdala slices revealed that the repeated delivery of blue light (465 nm at 20 Hz) generated a robust and high-fidelity firing of those CA1 and BLA neurons expressing ChR2-EYFP (Figures 2G–2J). By contrast, light stimulation had no effect on the membrane potential or firing of cells expressing only EYFP (Figures S2D–S2G). In addition, expression of endogenous c-Fos was induced in ChR2-expressing cells compared to EYFP-expressing cells at both of CA1 and BLA (Figure S2H).

Synchronous Activation of Distinct Cell Ensembles Creates an Artificial Association of Corresponding Information

Taking advantage of the specific expression of ChR2 and light-induced neuronal firing, we examined whether the simultaneous activation of distinct cell ensembles corresponding to the context pre-exposure and the IS in the unpaired condition could generate an artificial association between these two previously unassociated events. We implanted guide cannulae and injected TRE::EYFP or TRE::ChR2-EYFP LV into c-*fos*::tTA mice, allowing the mice at least 9 days to recover (Figures 3A and S3A). The mice were then taken off Dox for 2 days to allow for neuronal activity-dependent transgene expression and then conditioned

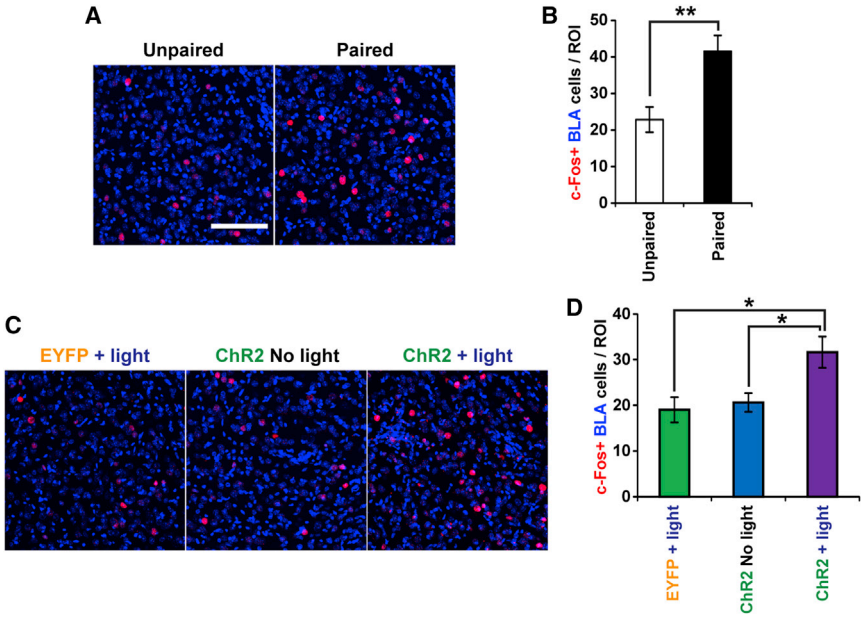


Figure 4. Both Artificial and Physiological Associations Increase the Representation of Sensory Input from the Context in the BLA Circuit

(A and B) Endogenous c-Fos expression in the BLA of mice that experienced paired or unpaired conditioning, as shown in Figure 1A.

(C and D) Endogenous c-Fos expression in the BLA of mice that underwent EYFP + light, ChR2 no light, or ChR2 + light, as shown in Figure 3A. (A and C) Representative images showing c-Fos (red) and DAPI nuclear staining (blue). Scale bar, 100 μm. (B and D) Graphs showing the number of c-Fos-positive cells in the regions of interest (ROI) within the BLA. (B) n = 6 sections from three mice/group; $^{**}p < 0.01$, unpaired Student's t test, two tailed. (D) n = 8 sections from four mice/group; $p < 0.01$, one-way ANOVA; $^{*}p < 0.05$, Scheffe's post hoc multiple comparisons test. Error bars, mean ± SEM.

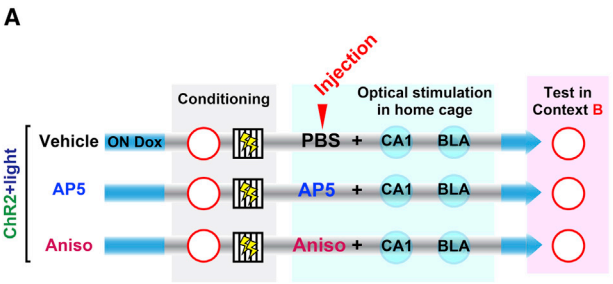
to the unpaired paradigm (in which naive mice did not make an association between the pre-exposed context B and an IS in context A) (Figures 1A and S3B). One day after conditioning, we delivered 20-Hz light pulses for 2 min to both CA1 and BLA (+ light) in the home cage (ChR2 + light group in Figure 3B). Control mice were attached to optic fibers targeting CA1 and the BLA, but the light pulse was not delivered (ChR2 no-light group in Figure 3B). The TRE::EYFP LV-injected mice served as another control group in which light pulses were delivered to both the CA1 and BLA (EYFP + light group in Figure 3B). Mice were then subjected to a fear memory test in the pre-exposed context B or C (Figure 3A).

When light stimulation was delivered and TRE::ChR2-EYFP LV-injected mice were tested in the pre-exposed context B, in which they had not experienced a foot shock, the mice showed a freezing level nearly equal to that of mice exposed to the paired condition (ChR2 + light in Figure 3B; see also Figures 1A, S3A, and S3B). By contrast, even when light stimulation was delivered, the group of TRE::EYFP LV-injected mice showed a significantly lower level of freezing than the TRE::ChR2-EYFP LV-injected mice, and this lower level was similar to that shown by mice in the unpaired group shown in Figure 1A (EYFP + light group in Figure 3B). A similarly low level of freezing was also observed in TRE::ChR2-EYFP LV-injected mice that did not receive light stimulation (ChR2 no light group in Figure 3B). The freezing level of mice in the ChR2 no-light groups was significantly lower than that of mice in the ChR2 + light group (Figure 3B). The mice in the ChR2 NoS + light group that did not undergo IS showed extremely low levels of freezing, indicating that the IS indeed acted as an unconditioned stimulus in this behavioral paradigm.

The high freezing level observed in mice in the ChR2 + light group was context B specific because freezing in a novel context C, a neutral context for them (Figures S1A, S1B, and S3B), was comparable to that observed for mice in the control groups and EYFP + light in context C group (Figure 3B). The context specificity was further confirmed by the observation that mice in the ChR2 + light group showed significantly more freezing in context B than in context A (Figures S3C and S3D). Therefore, the artificial associative memory contains a specific linkage to cell ensembles corresponding to the original contextual information (context B). The delivery of light pulses alone to the CA1 or BLA failed to increase freezing behavior (ChR2 + CA1 light and ChR2 + BLA light groups in Figure 3B) compared with that in the control groups. These results indicate that the synchronous activation of distinct cell ensembles residing in the CA1 and BLA, even 1 day after training, establishes a strong association between information corresponding to the pre-exposed context and IS that is comparable to memory conditioned with the paired paradigm.

Cell Ensemble Size in the BLA Increases during Physiological or Artificial Association Memory Processing

The number of c-Fos-positive cells in the BLA of mice in the paired group 1.5 hr after the test session was significantly higher than that in mice from the unpaired group (Figures 4A and 4B). This result indicates that the number of cells recruited in a recall process increases in the BLA when an association between the pre-exposed context and the IS is successfully established. Similar results were reported for cells in the BLA after the recall of a conventionally induced contextual fear memory (Ramirez et al., 2013; Reijmers et al., 2007). Therefore, we examined the pattern of c-Fos expression in the BLA of control mice (EYFP + light and ChR2 no-light groups) and mice in the ChR2 + light group after the memory test (Figure 4C). The number of c-Fos-positive cells in the ChR2 + light group increased significantly compared with that in control mice (Figure 4D); thus, similar to a physiological association, the size of the activated cell ensemble in the BLA increased in response to the synchronous light stimulation that generated an artificial association between the context and the IS.



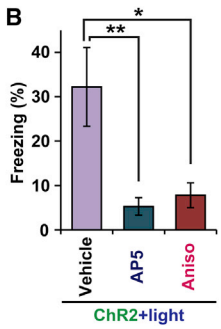


Figure 5. Artificial Association Requires N-Methyl-D-Aspartate Receptor Activity and De Novo Protein Synthesis during Optical Stimulation

(A) Diagram showing the experimental scheme. Inhibition of protein synthesis and NMDAR activity were performed with bilateral microinfusion of 0.5 μl of 125 μg/μl anisomycin (Aniso) and 30 mM D-AP5, respectively, into the CA1 and the BLA. Phosphate buffered saline (0.5 μl) was used as the vehicle. The microinfusion was conducted 20 min before the optical stimulation.

(B) Graph showing the freezing response during the memory test in context B ($p < 0.004$, one-way ANOVA; *$p < 0.05$, **$p < 0.01$, Scheffe's post hoc multiple comparisons test). Error bars, mean ± SEM.

Establishing an Artificial Association Depends on NMDAR Activity and Protein Synthesis

To gain insights into the mechanisms underlying the artificial association, we next investigated the effects of D-APV, an NMDAR antagonist, on the artificial association. The microinjection of D-APV into both hippocampus and amygdala via the double-targeting cannula system before the optical stimulation significantly reduced the expression of freezing behavior in the test session compared with that observed following vehicle injection (Figure 5), suggesting the involvement of Hebbian plasticity in the artificial association (Caporale and Dan, 2008; Fell and Axmacher, 2011; Hebb, 1949). Anisomycin, a protein synthesis inhibitor, elicited a similar effect on the freezing response (Figure 5), suggesting the involvement of a consolidation or reconsolidation mechanism (Abel et al., 1997; Hardt et al., 2010; Nader and Hardt, 2009; Nader et al., 2000). These results indicate that the establishment of associative memories generated under either physiological or artificial conditions share common molecular mechanisms.

Temporal Changes in Artificial and Physiological Associative Memories Are Comparable

Contextual fear memory becomes generalized with the passage of time (usually 2–4 weeks after conditioning in mice) such that the context specificity of the fear response decreases and animals exhibit increased freezing behavior in a novel context (Kitamura et al., 2012; Wiltgen and Silva, 2007). We investigated temporal changes in the physiological and artificial associative memories using mice in the naive paired and ChR2 + light groups, respectively (Figure 6A). In the recent memory phase, both groups conditioned with the paired paradigm showed specificity in freezing to the conditioned context (Figure S3B, compare lines 2 and 4 for naive paired; Figure 3 for ChR2 + light). In addition, the freezing response for both groups was comparable in the test of recent memory (Figure 6B). After 28 days, both groups were tested for freezing in the novel context C (remote memory test 1). No difference was observed in the freezing responses between the groups (Figure 6C). The next day, animals were tested in the pre-exposed context, i.e., context A for mice in the naive paired group and context B for those in the ChR2 + light group (remote memory test 2). We again observed a similar freezing response in both groups (Figure 6D). Freezing behavior in either group was not significantly altered during these tests, even though remote memory test 1 was performed in a novel context (Figure S4). Therefore, for the artificially associated memory, the recent memory phase was context specific but the remote phase lost the specificity, indicating that it underwent generalization. This feature is shared with physiologically associated memory, e.g., the paired paradigm.

DISCUSSION

Here, we showed that simultaneous optical stimulation of the subset of cells in the CA1 and BLA activated during initial learning associated two initially independent events. Therefore, the entirely artificial activation of two separate sets of neuronal ensembles, in the absence of new sensory input from conditioned or unconditioned stimuli (the context pre-exposure and foot shock), is capable of connecting two independent events. This artificially associated memory shared several characteristics with a physiologically associated memory: both depended on NMDAR activity and protein synthesis, both were long lasting (at least 4 weeks), and both underwent generalization. Previous studies showing synthetic or false memory generation by artificially activating a set of cell ensembles utilized sensory information for the unconditioned stimulus (Garner et al., 2012; Ramirez et al., 2013) or for the sensory cue (Johansen et al., 2010). These studies employed a combination of artificial stimulation of cell ensembles and sensory input. Therefore, to the best of our knowledge, our study is the first to link two distinct memories using wholly artificial means.

The size of the cell ensemble that corresponded either to the experience with the pre-exposed context or the IS, as determined by the number of *arc*-positive cells, was equivalent for the paired and unpaired conditions in both the CA1 and BLA; however, the size of the cell ensembles corresponding to both experiences, as determined by the number of double-positive *arc* neurons, increased significantly in the paired condition (Figures 1D and 1G). These findings suggest that overlap between the two ensembles contributes to the association of the two events. An ensemble appears to have the characteristic of stronger synaptic efficacy among the neurons within the ensemble compared with other neurons not belonging to the ensemble. The coincident activation of a set of neurons results in a strengthening in synaptic efficacy among these neurons (Caporale and Dan, 2008; Fell and Axmacher, 2011; Hebb, 1949). Therefore,

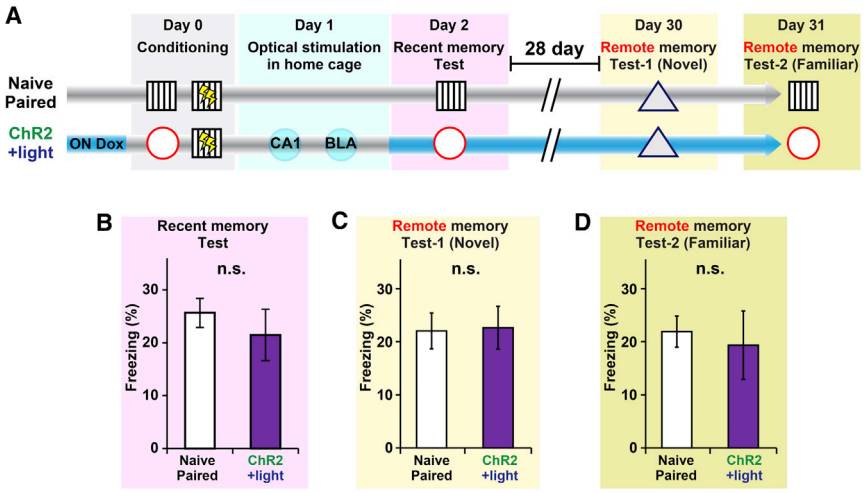


Figure 6. Temporal Changes in Artificial Associative Memory and Physiological Associative Memory Are Comparable

(A) Diagram showing the experimental scheme. The naive paired group (naive animals with no operations) was conditioned with the paired paradigm of context pre-exposure and immediate shock (IS). The ChR2 + light group was conditioned with the paradigm shown in Figure 3A.

(B–D) Graphs show freezing responses during each memory test. (B) Results of the test examining recent memory in pre-exposed contexts ($p > 0.4$, unpaired Student's t test, two tailed). (C) Results of the remote memory test 1 on day 30 in a novel context (context C) to examine the degree of generalization in fear memory ($p > 0.9$, unpaired Student's t test, two tailed). (D) Results of the remote memory test 2 on day 31 in pre-exposed familiar contexts ($p > 0.6$, unpaired Student's t test, two tailed). n.s., no significant difference between the two groups. Error bars, mean ± SEM.

See also Figure S4.

the synchronous light stimulation of the two cell ensembles in the present study likely strengthens the synaptic efficacy between the members of both ensembles within the CA1 and BLA, an assertion supported by the NMDAR dependence of the artificial association. This in turn leads to the formation of a cell ensemble integrating the two distinct types of information. The firing of one cell ensemble (context B) leads to the firing of the other cell ensemble (IS); thus, recall of the memory for context B triggers the recall of the IS memory and induces freezing behavior. We propose that optical stimulation forms a strong association in this manner.

The synchronous activation of both CA1 and BLA cell ensembles, which were specifically labeled with ChR2-EYFP in an OFF-Dox-dependent and neural activity-dependent manner (Figures 2D–2F), was required to generate the artificial association, because optical stimulation of either the CA1 or the BLA ensemble alone failed to generate the association (Figure 3). The catFISH analysis showed the existence of cell populations in both CA1 and BLA, which were activated by context pre-exposure or IS. Cells activated by contextual information predominantly appeared in CA1 (Figure 1C), whereas both contextual and IS information similarly impacted the BLA cell population (Figure 1F). Although the cell ensembles corresponding to the pre-exposed context and the IS could be merged, at least in part, by optical stimulation within each brain region, the lack of a synchronous activation of CA1 and the BLA failed to induce Hebbian plasticity between the CA1 and BLA ensembles. A direct anatomical interaction between CA1 in the dorsal hippocampus and the BLA has not been demonstrated; however, a functional interaction exists between CA1 and the lateral amygdala, as demonstrated by theta rhythm synchronization during fear memory retrieval, which is implicated in the promotion of synaptic plasticity (Seidenbecher et al., 2003). Therefore, indirect interactions between these two regions via other brain areas appears to be required to form a merged cell ensemble in which synchronized synaptic inputs and clustered synaptic plasticity may play important roles (Takahashi et al., 2012).

In our experiments, light stimulation was delivered 1 day after conditioning when mice were in their home cages. Optical stimulation of two cell ensembles is likely to trigger recall of the corresponding memories, which may lead to memory reconsolidation, a process requiring protein synthesis (Nader et al., 2000). Memory reconsolidation is assumed to underlie memory updating that, by destabilizing the original memory, integrates new and existing information (Hardt et al., 2010; Lee, 2009; Nader and Hardt, 2009; Tronson and Taylor, 2007). Integrating the two ensembles and reconsolidation mechanisms upon memory recall may both contribute to linking the pre-exposed context with the IS.

The duration of synchronous activity between the CA1 and BLA cell ensembles may be important for the generation of an artificial association. A previous study showed that the optical reactivation of CA1 neurons previously activated in a particular context during fear conditioning failed to create a false memory (Ramirez et al., 2013). In that study, the duration of the synchronous activity, consisting of CA1 light stimulation and an external unconditioned stimulus input, was partially overlapped and extremely short time, only 2 s each in three times. Long-term synchronization lasting for minutes, as induced n the present study, may be important for the stabilization of the functional interaction between cell ensembles in these regions. It will be of particular interest to define the conditions, such as the spatial and temporal correlates of the two ensemble activities, under which animals successfully associate an unconditioned stimulus with a conditioned stimulus in fear memory paradigms.

EXPERIMENTAL PROCEDURES

Full details are provided in the Supplemental Experimental Procedures.

Virus Injection

The pLenti-TRE::EYFP and pLenti-TRE::ChR2-EYFP plasmids were constructed and used for LV preparation as described previously (Goshen et al., 2011). The LVs were stereotaxically injected into the right hemisphere of the CA1 and BLA in c-*fos*::tTA mice.

Ex Vivo Electrophysiology

Transverse brain slices were prepared and the membrane potentials of ChR2-EYFP-expressing neurons in the CA1 and BLA regions were recorded under whole-cell current-clamp mode. Light stimulation (465 nm; 20-Hz pulse train for 2 s, total of 40 pulses; pulse duration, 5 ms) was delivered with a high-power LED illumination system.

Histology

The *arc* catFISH analysis was performed as described in previous studies (Guzowski et al., 1999; Hashikawa et al., 2011). For immunohistochemistry, brain sections were incubated with anti-GFP or anti-c-Fos antibodies followed by incubation with secondary antibodies and confocal microscopy.

Behavioral Analysis

Mice were pre-exposed to a context for 6 min, returned to their home cages for 30 min, and then subjected to a 0.8 mA foot shock for 2 s in context A after 5 s of acclimation. The unpaired paradigm used distinct contexts for pre-exposure (context B) and the IS in context A. The paired paradigm used the same context (context A) for pre-exposure and the IS. The memory tests for the unpaired and paired conditions were performed in contexts B and A, respectively. Cannula-implanted and LV-injected c-*fos*-tTA mice were maintained on Dox food pellets (40 mg/kg). Dox was removed from the mice for 2 days, and then mice were trained in the unpaired paradigm (except for the mice in some groups). After 24 hr, the mice were anaesthetized for approximately 4 min, and optic fibers were attached through their cannulae. The mice were returned to their home cages for 20–25 min before optical stimulation (473 nm light, 20 Hz, 10 ms) was delivered in the home cage for 2 min. Five minutes after the end of the stimulation, the optic fiber was detached, and the mice were returned to their home cages. The mice were placed in several contexts for the fear memory retention test.

All behavioral experiments were conducted using a video tracking system (Muromachi Kikai) automated to measure the freezing behavior of the animals, as in a previous study (Kitamura et al., 2009). Freezing was defined as a complete absence of movement except for respiration. The duration of the freezing response was scored, beginning at 1.5 s of sustained freezing. The percentage of time spent freezing in the first 2 min of each test session was then averaged for all mice within each group. In contexts A, B, and C, no significant difference was found in the level of freezing for the mice during their first exposure (Figures S1A and S1B).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.03.017.

AUTHOR CONTRIBUTIONS

N.O. and K.I. designed the study. N.O. and E.M. cloned the lentiviral constructs. N.O. prepared the lentiviruses. S.T., H.N., and M.M. contributed to the production and maintenance of the transgenic animals. N.O. and Y.S. performed all animal surgery. N.O. and A.S. contributed to the behavioral experiments. N.O., A.S., S.T., and S.K. performed the histology and analysis. Y.T., M.N., Y.K.S., A.M.W., and F.K. conducted the electrophysiology experiments. N.O. and K.I. wrote the manuscript.

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