



## Supplementary Material for

### **Creating a False Memory in the Hippocampus**

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## **Materials and methods**

### **Subjects**

The *c-fos-tTA* mice were generated by crossing TetTag (*S1*) mice with C57BL/6J mice and selecting those carrying the *c-fos-tTA* transgene. Mice were group-housed with littermates until the beginning of the surgery and given food and water *ad libitum*. The mice were 8–14 weeks old at the time of surgery and had been raised on a diet containing 40 mg kg<sup>-1</sup> doxycycline for a minimum of 1 week before surgery. Mice were housed individually post-surgery and throughout the duration of the experiments. All procedures relating to mouse care and treatment conformed to the institutional and National Institutes of Health guidelines.

### **Virus constructs**

The pAAV-TRE-ChR2-mCherry plasmid was constructed by replacing the *EYFP* sequence in the pAAV-TRE-ChR2-EYFP plasmid (*S2*) with the sequence for *mCherry* using AgeI and BsrGI restriction sites. The pAAV-TRE-mCherry plasmid was constructed by removing the *ChR2* fragment from the pAAV-TRE-ChR2-mCherry plasmid using NheI and AgeI restriction sites, blunting with T4 DNA polymerase, and self-ligating the vector, which retained the ATG start codon of the *mCherry* gene from the *ChR2-mCherry* fusion gene. These plasmids were used to generate AAV<sub>9</sub> viruses by the Gene Therapy Center and Vector Core at the University of Massachusetts Medical School. Viral titers were  $8 \times 10^{12}$  GC/ml for AAV<sub>9</sub>-TRE-ChR2-mCherry and  $1.4 \times 10^{13}$  GC/ml for AAV<sub>9</sub>-TRE-mCherry. Viral titers were  $1 \times 10^{13}$  GC/ml for AAV<sub>9</sub>-TRE-ChR2-EYFP and  $1.5 \times 10^{13}$  GC/ml for AAV<sub>9</sub>-TRE-EYFP as previously reported (*S2*).

### **Stereotactic injection and optical fiber implant**

All surgeries were performed under stereotaxic guidance. Mice were anaesthetized using 500 mg kg<sup>-1</sup> Avertin. Each animal underwent bilateral craniotomies using a 0.5 mm diameter drill bit at -2.2 mm anteroposterior (AP),  $\pm 1.3$  mm mediolateral (ML) for DG injections; -2.0 mm AP,  $\pm 1.5$  mm ML for CA1

injections (S3). The virus was injected using a mineral oil-filled glass micropipette joined by a microelectrode holder to a 10  $\mu\text{l}$  Hamilton microsyringe. A microsyringe pump and its controller were used to control the speed of the injection. The needle was slowly lowered to the target site ( $-2.0$  mm dorsoventral (DV) for DG injections;  $-1.2$  mm DV for CA1 injections) and remained for five min before the beginning of the injection. All mice were injected bilaterally with  $0.15$   $\mu\text{l}$  AAV<sub>9</sub> virus at a rate of  $0.6$   $\mu\text{l min}^{-1}$ . The micropipette was kept at the target site for another five minutes post-injection before being slowly withdrawn. After withdrawing of the needle, a bilateral patch cord optical fiber implant ( $200$   $\mu\text{m}$  core diameter) was lowered above the injection site ( $-1.6$  mm DV for DG;  $-1.0$  mm DV for CA1). A miniature screw was screwed securely into the skull at the anterior and posterior edges of the surgical site to provide two extra anchor points for the implant. A layer of adhesive cement was applied to secure the optical fiber implant to the skull. A protective cap made from the top portion of a black polypropylene microcentrifuge tube was used to encircle the surgical site, and dental cement was applied to secure the cap to the implant and close up the surgical site. Each animal was given  $1.5$   $\text{mg kg}^{-1}$  analgesics via intraperitoneal injection and remained on a heating pad until fully recovered from anesthesia. All mice were allowed to recover for two weeks before all subsequent experiments. All fiber placements and viral injection sites were verified histologically. We only included mice in this study that had opsin or fluorophore expression limited to either DG or to CA1.

### **Slice recordings**

Mice (P30–P35) were anesthetized by isoflurane, decapitated and brains were quickly removed. Sagittal slices ( $300$   $\mu\text{m}$  thick) were prepared by using a vibratome in an oxygenated cutting solution at  $\sim 4$   $^{\circ}\text{C}$ . Slices were then incubated at room temperature ( $\sim 23$   $^{\circ}\text{C}$ ) in oxygenated ACSF until the recordings. The cutting solution contained (in mM): 3 KCl, 0.5 CaCl<sub>2</sub>, 10 MgCl<sub>2</sub>, 25 NaHCO<sub>3</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 10 D-glucose, 230 sucrose, saturated with 95% O<sub>2</sub> – 5% CO<sub>2</sub> (pH 7.3, osmolarity 300 mOsm). The ACSF contained (in mM): 124 NaCl, 3 KCl, 2 CaCl<sub>2</sub>, 1.3 MgSO<sub>4</sub>, 25

NaHCO<sub>3</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 10 D-glucose, saturated with 95% O<sub>2</sub> – 5% CO<sub>2</sub> (pH 7.3, osmolarity 300 mOsm). Individual slices were transferred into a submerged experimental chamber and perfused with oxygenated ACSF warmed at 36 °C (±0.5 °C) at a rate of 3 ml/min during recordings. Whole cell recordings in current clamp or voltage clamp mode were performed by using an IR-DIC microscope mounting a water immersion 40× objective (NA 0.8), equipped with four automatic manipulators and a CCD camera. For all the recordings borosilicate glass pipettes were fabricated with resistances of 8 to 10 MΩ, and filled with the following intracellular solution (in mM): 110 K-gluconate, 10 KCl, 10 HEPES, 4 ATP, 0.3 GTP, 10 phosphocreatine and 0.5% biocytin. The osmolarity of this intracellular solution was 290 mOsm and the pH was 7.25. Access resistance (Ra) was monitored throughout the duration of the experiment and data acquisition was suspended whenever the resting membrane potential was depolarized above –50 mV or the Ra was beyond 20 MΩ. Recordings were amplified using up to two dual channel amplifiers, filtered at 2 kHz, digitized (20 kHz), and acquired using custom made software running on Igor Pro. Optogenetic stimulation was achieved through a 460 nm LED light source driven by TTL input with a delay onset of 25 μs (subtracted off-line for the estimation of the latencies). Light power on the sample was 33 mW/mm<sup>2</sup>. Slices were stimulated by a train of twenty 15 ms light pulses at 20 Hz every 5 s. In voltage clamp mode cells were held at –70 mV for EPSC measurements while in current clamp mode, EPSP and APs were measured at resting potentials.

### **Head-fixed recording**

Mice were anaesthetized by injection (100 ml kg<sup>-1</sup>) of a mixture of ketamine (100 mg ml<sup>-1</sup>) / xylazine (20 mg ml<sup>-1</sup>) and placed in the stereotactic instrument with anaesthesia maintained with a series of ketamine boost (100 mg ml<sup>-1</sup>) throughout the recording. Body temperature was maintained by a pack of Hand Warmers. An optrode consisting of a tungsten electrode (0.5 MΩ) attached to an optical fiber (200 μm core diameter), with the tip of the electrode extending beyond the tip of the fiber by 300 μm, was used for simultaneous optical stimulation and

extracellular recordings. The optrode was slowly lowered to the DG (AP -2.2 mm; ML +1.3 mm; DV -2.0 mm) using a hydraulic micropositioner at a speed of 50  $\mu\text{m}$  per 5–10 min. The optical fiber was connected to a 200 mW 473 nm blue laser and controlled by a waveform generator. The power intensity of light emitted from the optrode was calibrated to about 7 mW, which was consistent with the power intensity used in the behavioral assays. To identify ChR2-labelled cells, light pulses of 15 ms were delivered at 0.2 Hz at the recording sites approximately every 50  $\mu\text{m}$  throughout the DG. After light responsive cells were detected, two types of light stimuli were tested: 15 ms light pulse every 5 s and a train of ten 15 ms light pulses at 20 Hz every 5 s. Unit activity was band-pass filtered (500 Hz–5 kHz) and acquired with an Axon Digidata 1440A acquisition system running Clampex 10.2 software. Data were analyzed with custom software written in Matlab. After the recording, endogenous c-Fos expression was induced by delivering two epochs of 3-min light stimulation (7 mW, 20 Hz, 15 ms), separated by 3 min, to the DG, the same as in behavioral experiments (see below). Mice were sacrificed and perfused 90 min later.

### **Immunohistochemistry**

Mice were overdosed with 750–1000 mg kg<sup>-1</sup> Avertin and perfused transcardially with cold PBS, followed by 4% paraformaldehyde (PFA) in PBS. Brains were extracted from the skulls and kept in 4% PFA at 4 °C overnight, then transferred to PBS. Fifty  $\mu\text{m}$  coronal slices were taken using a vibratome and collected in cold PBS. For immunostaining, each slice was placed in PBS-T (PBS + 0.2% Triton X-100) with 5% normal goat serum for 1 h and then incubated with one or more primary antibodies (1:1000 dilution) at 4°C for 24 h (600-401-379 Rockland; A10262, Invitrogen; SC-52, Santa Cruz). Slices then underwent three wash steps for 10 min each in PBS-T, followed by 1 h incubation with secondary antibody at 1:200 dilution (A11039, Invitrogen; A21429, Invitrogen). Slices then underwent three more wash steps of 10 min each in PBS-T, including DAPI (1:10000 dilution) in the first wash step, followed by mounting with Vectashield H-1200 and coverslipping on microscope slides.

## Behavior assays

All behavior tests were administered during the light cycle (7 am –7 pm) of the day. Contextual fear conditioning assays were conducted in one of the four distinct contexts (A, B, C, and D). Context A was a 29 × 25 × 22 cm chamber with removable black cardboard floor and scented with 1% acetic acid from a tray underneath. Within each chamber was a black plastic triangular roof. Context A was located underneath two lamps emitting red light in a room with dim lighting. Context B was a 30 × 25 × 33 cm chamber with metal gridded floor and scented with 0.25% benzaldehyde. It is located in a second room with black walls, black curtains, and intermediate lighting. Context C was a 29 × 25 × 22 cm chamber with glossy white plastic floor and scented with 1 ml of citral in a tray underneath the floor. It is in a third room with bright light distinct from context A and B. Context D was a 32 × 25 × 27 cm unscented chamber with matte white plastic floor within a 64 × 73 × 40 sound attenuating cubicle with internal lightings. It is located in a fourth room distinct from contexts A, B, and C.

Prior to the behavioral experiments, all mice were handled for five days. They were taken off Dox for 42 hours to open a window of activity-dependent labeling. They were then placed in context A and allowed to explore for ten minutes, after which they were immediately removed from the chamber and placed on 40 mg kg<sup>-1</sup> Dox diet to shut off further labeling. Twenty-four hours later, mice were individually placed into context B and plugged to a complimentary optical fiber patch cord, which was connected to a 473 nm laser under the control of a function generator. Mice were trained in context B for a total of 420 s. They were first allowed to explore context B for 120 s, after which blue light was administered (20 Hz, 15 ms pulse width, ~7–15 mW output from fiber tip) for the remaining 300 seconds. At 240 s into training, three mild foot shocks (0.75 mA) lasting 2 seconds each were administered with a sixty-second inter-shock interval. At the 420 s mark, mice were immediately removed from context B and placed back into their home cages. All post-training tests in contexts A (A') and C (C') consisted of three-minute exposure to the contexts. Test trials during re-exposure to context B (B') or

exposure to context D each lasted six minutes, beginning with a three-minute light-off epoch followed by a three-minute light-on epoch, with the same light stimulation parameters as the training session. Separate cohorts of mice underwent a similar behavioral schedule but were pre-exposed to context C for 10 min while on Dox a day after the first exposure to context A (Fig. 2G, N). The immediate shock group underwent the same behavioral protocol described for the group in which context A was labeled, except that training in context B lasted for 10 s with light stimulation, at the end of which a single 0.75 mA shock was administered for 2 s. While mice were in contexts A and C, freezing behavior was continuously recorded with a digital camera and measured with FreezeFrame software. Light stimulation during training on context B, re-exposure to context B (B'), or exposure to context D interfered with the motion detection of the program. To circumvent this issue, freezing during these sessions was manually scored by two experimenters in a double-blind fashion. The manual scoring and automated scoring yielded freezing scores with a difference of less than 5%.

For the conditioned place avoidance (CPA) experiments, the CPA apparatus consisted of two 15 × 15 × 20 cm chambers (A and B) connected by a triangular neutral zone (15 cm for each side). Chamber A consisted of black and white striped walls and contained a transparent floor with small irregular indentations. Chamber B consisted of black and white alternating polka dotted walls and contained a smooth plastic floor. The mice did not have an innate preference for either portion of the apparatus (fig. S6A). Experimental mice were first taken off Dox for 42 hours to open a window of activity-dependent labeling. They were then exposed in a counterbalanced manner to either chamber A or B (labeled chamber) for ten minutes to label the cells active in the respective chamber. Then they were placed back on Dox diet and exposed to the other chamber (unlabeled chamber) 24 h later. These mice then underwent the same fear conditioning protocol with light stimulation in context B as described above. Twenty-four hours later, all groups were placed in the neutral zone of the CPA apparatus and preference scores were measured continuously across a twelve-minute session by automated scoring software. To calculate preference scores, we divided the total amount of time that

each animal spent in the unlabeled chamber by the total amount of time it spent in the labeled chamber. Thus a value above 1 indicates a preference for the unlabeled chamber; and a value below 1 indicates a preference for the labeled chamber. Moreover, to calculate difference scores, we subtracted the total amount of time each animal spent in the unlabeled chamber by the total amount of time the animal spent in the labeled chamber.

### **Cell counting**

To measure the extent to which populations of active cells overlap between the exposure to the same or different contexts, we counted the number of mCherry and c-Fos immunoreactive neurons in DG and CA1 from five coronal slices (spaced 160  $\mu\text{m}$  from each other) per mouse ( $n = 4$  for all groups). These slices were taken from dorsal hippocampus and focused on the coordinates that our injection and optical fiber implants targeted ( $-1.94$  mm to  $-2.74$  mm AP). Fluorescence images were acquired using a microscope with a  $\times 20/0.50$  NA objective. Mice injected with AAV-TRE-ChR2-mCherry in DG and CA1 were first taken off Dox for 42 hours to open a period of activity-dependent labeling. They were then placed in context A for ten minutes to label the cells active in this environment and placed on Dox immediately following the session. The next day, half of the mice were placed back in context A (the A-A group) and half were placed in a distinct context C (the A-C group). Both groups were sacrificed 1.5 h later for immunohistochemistry analyses. The overlap between mCherry and c-Fos in these experiments was quantified with ImageJ (S4). Background autofluorescence was removed by applying an equal cutoff threshold to all images by an experimenter blind to experimental conditions. Statistical chance was calculated by multiplying the observed percentage of mCherry-single-positive cells by the observed percentage of c-Fos-single-positive-cells.

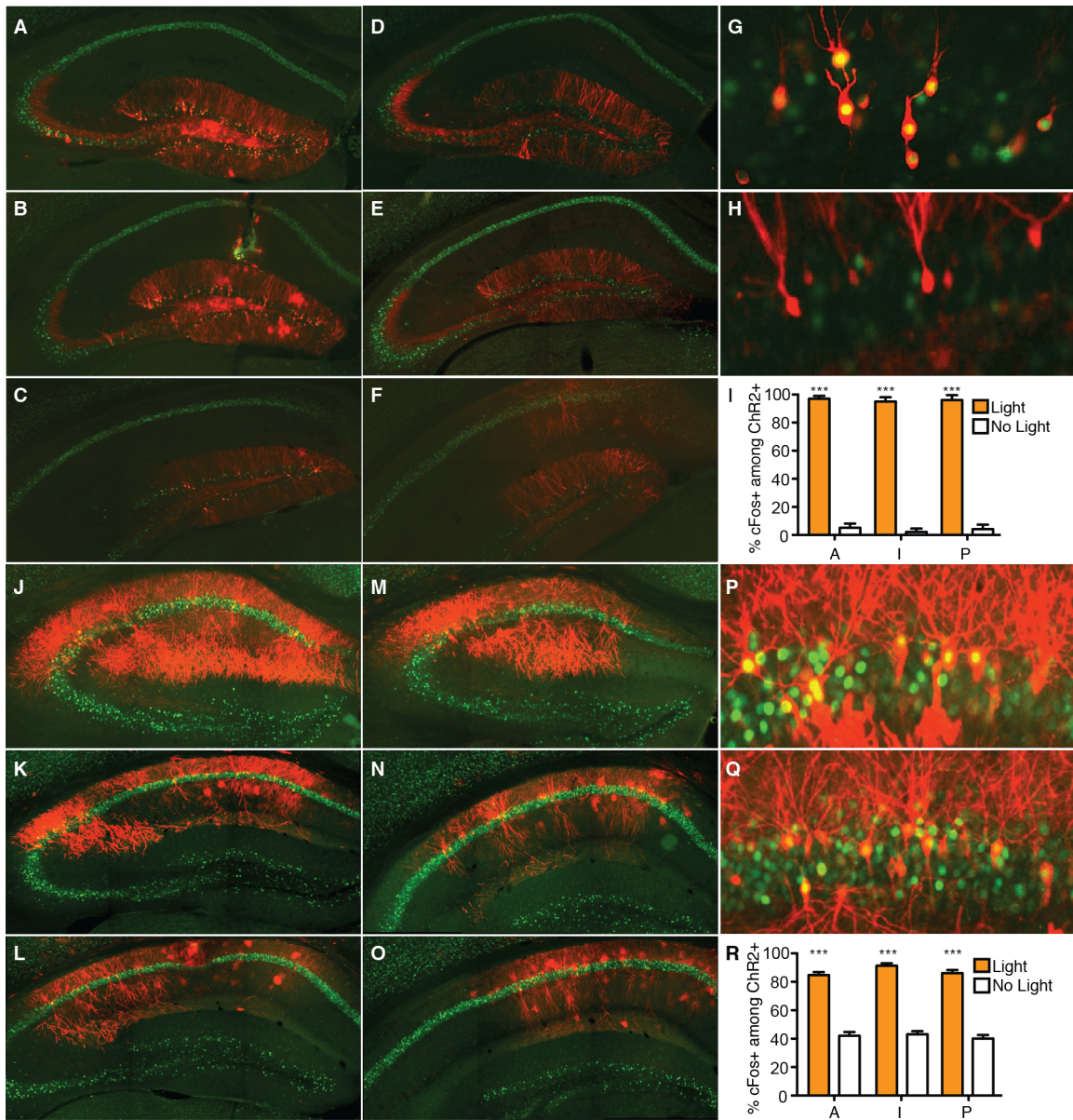
To measure the extent to which false and genuine memories engage similar brain regions, mice were taken off Dox for 42 hours and then exposed to context A for ten minutes to label DG cells with ChR2-mCherry. The following day, they were



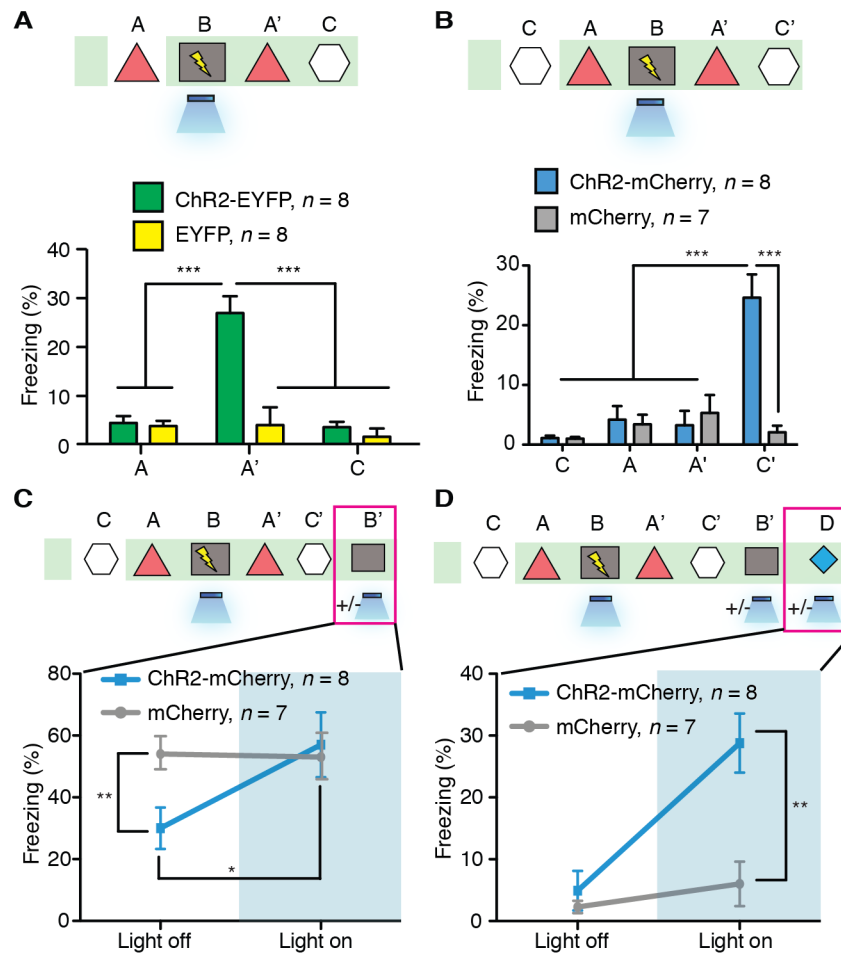
exposed to context C while on Dox for ten minutes. These mice were then divided into three groups: two groups underwent fear conditioning in context B with light stimulation as described above, and one group with no light stimulation during fear conditioning. For the first two groups, one group was then re-exposed to context C (C') and sacrificed 1.5 h later. The other group was re-exposed to context A (A') for a false memory test and sacrificed 1.5 h later. The third group, which did not receive light during fear conditioning, was re-exposed to context B (B') for natural fear memory recall, and sacrificed 1.5 h later.

Automated cell counting of c-Fos-positive cells was performed in the amygdala by utilizing image analysis software. This module quantified the number of c-Fos-positive cells per section (5 coronal slices per mouse;  $n = 6$  mice per condition) by thresholding c-Fos immunoreactivity above background levels and by using DAPI staining to differentiate between nuclei. Our regions of interest (ROI) included the basolateral amygdala and central amygdala. Our sampled amygdala slices were spaced at least 40  $\mu\text{m}$  from each other and we focused on slices between -1.30 mm to -1.70 mm from Bregma. Each ROI was manually outlined for quantification. For statistical analysis, we used a one-way ANOVA followed by Tukey's multiple comparisons ( $\alpha = 0.05$ ). All data were analyzed and graphed using Microsoft Excel with the Statplus plug-in and Prism.

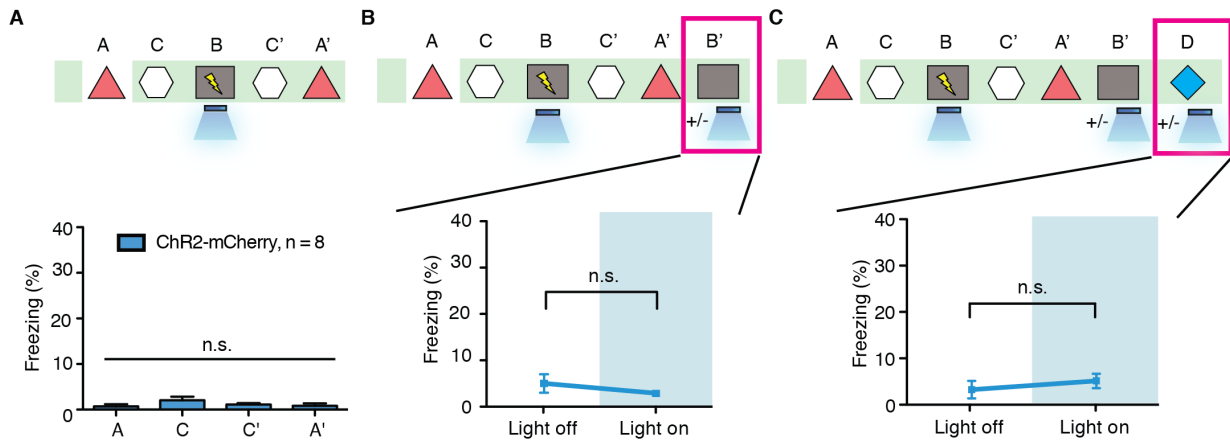
In a separate group of c-fos-tTA animals injected with AAV<sub>9</sub>-TRE-ChR2-mCherry targeted to either the DG or CA1, we determined the extent to which light activates cells along the anterior-posterior axis of these subregions. These groups were taken off Dox and exposed to context A for 10 min to induce ChR2-mCherry in DG or CA1. While back on Dox, light stimulation was administered (300 sec, ~9 mW, 20 Hz, 15 ms pulse width) the following day in context D and animals were sacrificed 1.5 hours later for histological analyses and quantification of cFos-positive cells using the immunohistochemistry protocol described above. The intermediate slices were defined as slices directly underneath the center of our optic fiber implant (-2.2 mm AP for DG and -2.0 mm AP for CA1) whereas anterior or posterior slices were selected 500  $\mu\text{m}$  away from the intermediate slices.



**Fig. S1.** Light stimulation induced cFos expression in ChR2+ cells throughout DG or CA1. (A–I) Animals expressing ChR2-mCherry (red) in the DG were treated with or without light stimulation and the expression of cFos (green) was examined. Representative anterior, intermediate, posterior, and higher magnification images of DG for the light stimulated group (A, B, C, and G) and no light group (D, E, F, and H) are shown. Quantification of cFos positive cells among ChR2 positive cells is shown in (I). A: anterior, I: intermediate, P: posterior. (n = 3/group; \*\*\* $P < 0.001$ ). (J–R) The same as A–I, except the ChR2-mCherry is expressed in CA1.

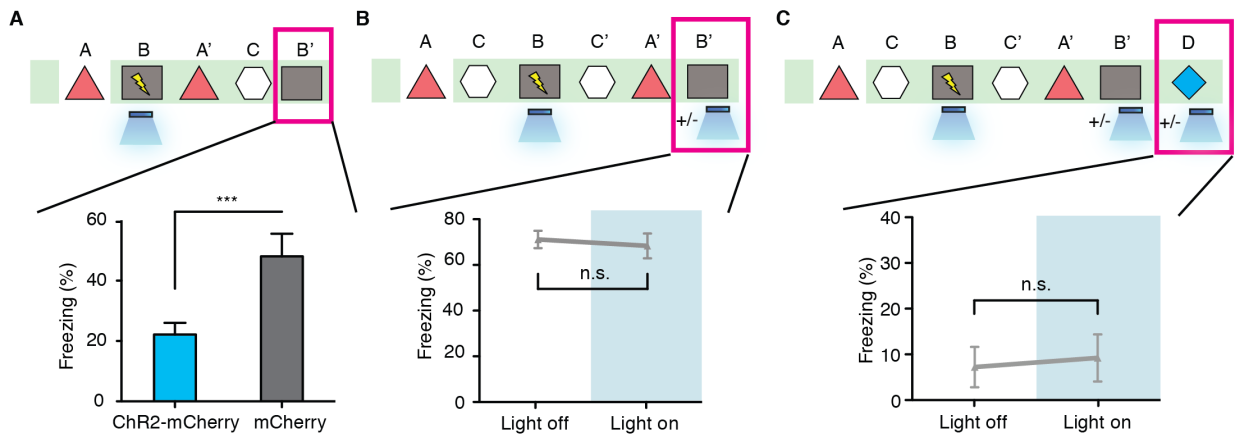


**Fig. S2.** Formation of false memory is reproducible under various conditions. **(A)** Top: c-fos-tTA animals injected with AAV<sub>9</sub>-TRE-ChR2-EYFP or AAV<sub>9</sub>-TRE-EYFP in DG underwent training and testing shown. Bottom: animals' freezing levels in context A before fear conditioning and in context A and C after fear conditioning ( $n = 8$  for each group;  $***P < 0.001$ ). **(B)** The c-fos-tTA animals injected with AAV<sub>9</sub>-TRE-ChR2-mCherry or AAV<sub>9</sub>-TRE-mCherry in DG that underwent the behavioral protocol shown above. The freezing levels for each session are shown ( $n = 8$  for ChR2-mCherry group and  $n = 7$  for mCherry group;  $***P < 0.001$ ). **(C and D)** The same animals from B were re-exposed to context B (C) and context D (D). The freezing levels were examined both in the absence and presence of light stimulation. ( $*P < 0.05$ ;  $**P < 0.01$ ).



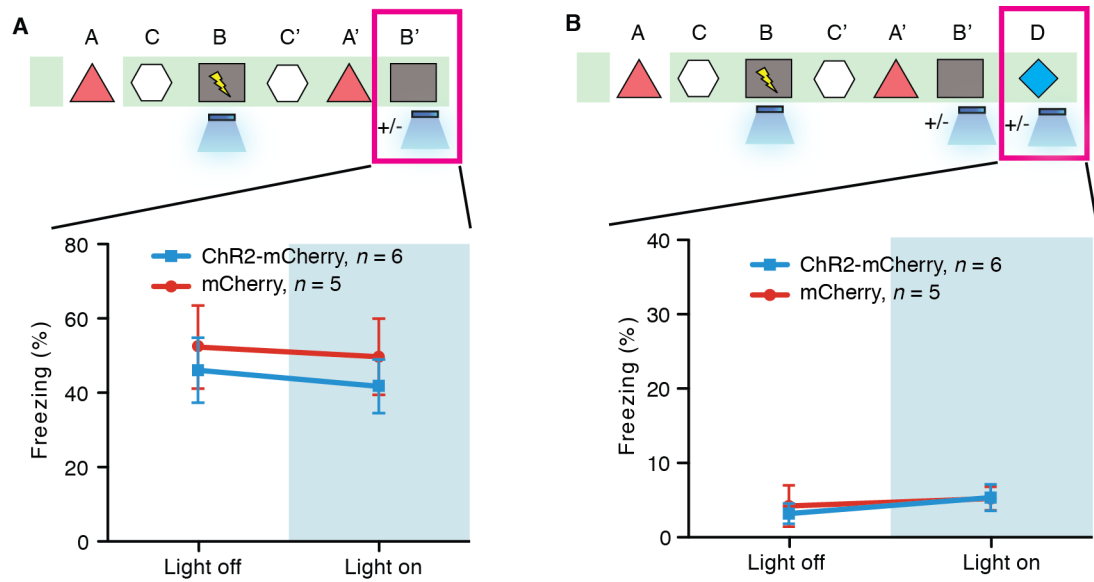
**Fig. S3.** Animals undergoing an immediate shock protocol with light stimulation do not form a false fear memory.

(A) The *c-fos*-tTA animals injected with AAV<sub>9</sub>-TRE-ChR2-mCherry in the DG underwent the behavioral protocol shown above and their freezing levels for each session are shown below ( $n = 8$  for ChR2-mCherry). (B and C) The same animals were re-exposed to context B and context D. Freezing levels were examined both in the absence and presence of light stimulation. (n.s.: not significant).



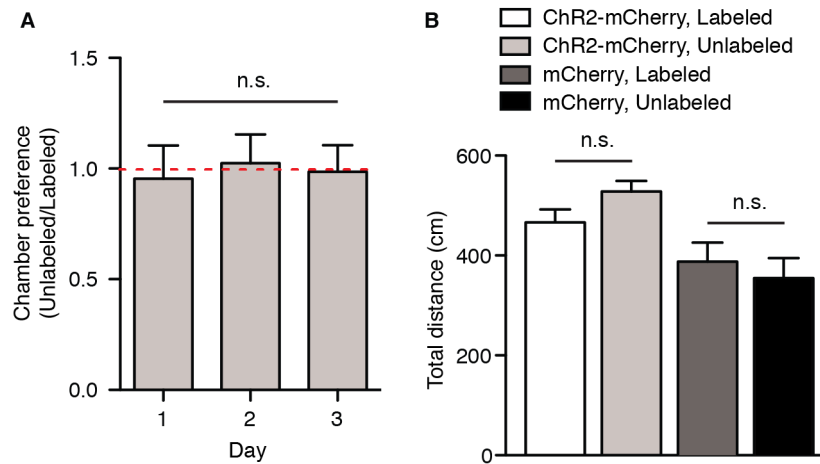
**Fig. S4.** False memory formation interfered with genuine memory recall.

(A) The c-fos-tTA animals injected with AAV<sub>9</sub>-TRE-ChR2-mCherry or AAV<sub>9</sub>-TRE-mCherry in DG that underwent the behavioral protocol shown above were re-exposed to context B and the freezing levels were measured. ( $n = 8$  for ChR2-mCherry group and  $n = 6$  for mCherry group;  $***P < 0.001$ ). (B and C) The c-fos-tTA animals injected with AAV<sub>9</sub>-TRE-mCherry in DG that underwent the behavioral protocol shown above were re-exposed to context B (B) and context D (C). The freezing levels were examined both in the absence and presence of light stimulation. ( $n = 6$ ; n.s.: not significant).



**Fig. S5.** Fear memory tests for CA1 animals in context B and context D.

The *c-fos*-tTA animals injected with AAV<sub>9</sub>-TRE-ChR2-mCherry or AAV<sub>9</sub>-TRE-mCherry in CA1 that underwent the behavioral protocol shown above were re-exposed to context B (**A**) and context D (**B**). The freezing levels were examined both in the absence and presence of light stimulation. ( $n = 6$  for ChR2-mCherry group and  $n = 5$  for mCherry group).



**Fig. S6.** Control data for the CPA experiments.

**(A)** A group of wild-type mice were exposed to the CPA apparatus and allowed to explore both chambers freely for 3 consecutive days. Their preferences for the chambers were measured as a ratio of time spent in each chamber during each day ( $n = 6$ ). **(B)** The *c-fos*-tTA animals injected with either AAV<sub>9</sub>-TRE-ChR2-mCherry or AAV<sub>9</sub>-TRE-mCherry in DG were taken off Dox and exposed to one chamber (Labeled) of the CPA apparatus, then put back on Dox and exposed to the other chamber (Unlabeled) the next day. Total distances traveled for each exposure were shown for each chamber for both groups ( $n = 8$  for each group).

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