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Pharmacogenetic reactivation of the original engram evokes an extinguished fear memory

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ABSTRACT

Fear memory extinction has several characteristic behavioral features, such as spontaneous recovery, renewal, and reinstatement, suggesting that extinction training does not erase the original association between the conditioned stimulus (CS) and the unconditioned stimulus (US). However, it is unclear whether reactivation of the original physical record of memory (i.e., memory trace) is sufficient to produce conditioned fear response after extinction. Here, we performed pharmacogenetic neuronal activation using transgenic mice expressing hM3Dq DREADD (designer receptor exclusively activated by designer drug) under the control of the activity-dependent *c-fos* gene promoter. Neuronal ensembles activated during fear-conditioned learning were tagged with hM3Dq and subsequently reactivated after extinction training. The mice exhibited significant freezing, even when the fear memory was no longer triggered by external CS, indicating that the artificial reactivation of a specific neuronal ensemble was sufficient to evoke the extinguished fear response. This freezing was not observed in non-fear-conditioned mice expressing hM3dq in the same brain areas. These results directly demonstrated that at least part of the original fear memory trace remains after extinction, and such residual plasticity might reflect the persistent memory.

1. Introduction

Persistency is a critical hallmark of memory. It describes the accurate, long-term storage of information. However, the persistency of fear memories can cause pathological maladaptations to environmental changes and result in anxiety disorders, such as phobias and post-traumatic stress disorder (PTSD). On the other hand, memories can be dynamically modified over time and by our daily experiences (Lacy and Stark, 2013). The fundamental question therefore arises as to whether the original internal representation of the memory (i.e., the memory trace) persists when the expression of that memory is altered (Dudai, 2012; Lütcke et al., 2013). This is a critical question for understanding the neural basis of memory and for the treatment of fear and anxiety disorders.

Pavlovian fear conditioning is a well-established model used to study the neural basis of fear memory (Fanselow and Poulos, 2005; Johansen et al., 2011). It involves association of a neutral conditioned stimulus (CS) with a noxious unconditioned stimulus (US), such as an electrical foot shock, and results in the acquisition of a persistent fear memory for the CS that manifests as a fear-related behavior. However, subsequent repeated re-exposure to the CS in the absence of the US eventually results in the loss of the fear-related behavior. This dynamic process, known as extinction, has several characteristic behavioral features, such as spontaneous recovery, renewal, and reinstatement, that favor the view that extinction does not erase the original association between the CS and the US (Myers and Davis, 2002; Maren and Quirk, 2004; Herry et al., 2010; Maren, 2011), thereby implying the existence of an extinction-resistant plasticity of the fear memory trace. Moreover, recent advanced genetic and physiological studies have begun to reveal neural circuits of fear memory and extinction memory embedded in the brain across several interacting regions such as the amygdala, prefrontal cortex, midbrain, and hippocampus. During extinction training, extinction networks that inhibit fear

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networks are considered to be formed. However, it is not clear whether the recovery of memory is caused by reactivation of the original neuronal ensembles activated during fear memory acquisition. We hypothesized that if plasticity of the neuronal ensemble that encoded the original fear memory trace was not abolished by extinction training, then forced activation of the neuronal ensembles would overcome extinction and lead to fear responses. To test this idea, here we attempted to examine whether forced activation of the original memory trace evokes the extinguished fear response. To manipulate the activities of memory traces sparsely distributed within brain tissue, we used a *c-fos* promoter driven tTA (tetracycline-controlled transactivator) transgenic system 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). The system has been used to provide a causal sufficiency and necessity between memory traces and the activities of specific ensembles of neurons that were activated during conditioned fear learning (Garner et al., 2012; Liu et al., 2012; Cowansage et al., 2014; Tanaka et al., 2014; Ohkawa et al., 2015; Matsuo, 2015).

2. Materials and methods

2.1. Subjects

The hM3Dq$^{c\text{-}fos}$ double transgenic mice were produced by mating cfos-tTA transgenic mice (Matsuo et al., 2008; Reijmers et al., 2007) with tetO-hM3Dq transgenic mice (Alexander et al., 2009). They were bred for more than 9 generations on the C57BL/6J background and maintained as heterozygotes. All mice were bred in social groups (2–5 mice per cage), provided food and water ad libitum, and fed a Dox diet (50 mg/kg) from the time of weaning (3–5 weeks old) to the onset of experiments (10–18 weeks old). To induce hM3Dq expression, Dox administration was discontinued for 9 days. Only males were used for behavioral experiments. All procedures were approved and conducted in accordance with guidelines of Kyoto University and Osaka University on the care and use of laboratory animals.

2.2. Immunohistochemistry

Brains were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C overnight and sectioned at a thickness of 40 μm using a vibratome (Leica). Free-floating slices were permeabilized with 0.3% TritonX-100 in 5% bovine serum albumin (BSA)/PBS at room temperature for 30 min, and then rinsed with PBS. We subsequently used enzyme antibody techniques for labeling c-Fos, and fluorescence antibody techniques for labeling HA, neuronal-specific nuclear protein (NeuN), and glutamate decarboxylase 67 (GAD67), a marker of GABAergic inhibitory neurons.

For enzyme antibody staining, permeabilized slices were incubated with the primary antibody (rabbit anti c-Fos antibody, 1:2,000, Ab-5, Calbiochem) at room temperature overnight. Next, slices were rinsed with 0.3% TritonX-100/PBS three times for 10 min, and incubated with the secondary antibody (biotin conjugated donkey anti-rabbit antibody, 1:500, AP182B, Millipore) at room temperature for 1 h. Slices were then rinsed with 0.3% TritonX-100/PBS three times for 10 min, and incubated with an avidin-biotinylated peroxidase complex reagent (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 7.6. Finally, slices were rinsed with PBS three times for 10 min and mounted with 50% glycerine.

For fluorescence antibody staining, permeabilized slices were incubated with primary antibodies (rabbit anti-HA antibody, 1:2,000, 600-401-384, Rockland; rat anti-HA antibody, 1:1,000, clone 3F10, Roche; rabbit anti-ZIF (Egr-1) antibody, 1:4,000, sc-189, Santa Cruz Biotechnology; mouse anti-NeuN antibody, 1:1,000, MAB377, Millipore; mouse anti-CaMKII antibody, 1:1,000, 05-532, Millipore; mouse anti-GAD67 antibody, 1:1,000, MAB5406, Millipore; rat anti-Ctip2 antibody, 1:2,000, ab18465, Abcam) at 4 °C overnight. Rat anti-HA antibody was used for double-staining of HA and Zif. Next, slices were rinsed with PBS three times for 10 min and incubated with secondary antibodies at 4 °C overnight (goat anti-rabbit AlexaFluor594, 1:800, Molecular Probes; goat anti-mouse AlexaFluor647, 1:800, Molecular Probes). Slices were then rinsed with PBS for 10 min and subsequently incubated with 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes) at room temperature for 3 min. Next, slices were rinsed with PBS three times for 10 min and mounted with SlowFade Gold antifade reagent (Molecular Probes).

For the quantification of c-Fos immunoreactivity, 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 c-Fos positive cells were counted by an experimenter blind to the conditions. Fluorescent images were obtained with an FV1000 confocal laser scanning microscope (Olympus) or an Axio Imager2 (Zeiss) equipped with an AxioCam MRm CCD camera (Zeiss).

2.3. Fear conditioning and extinction

All behavioral experiments were conducted during the light period of the light/dark cycle. At the start of experiments, mice were individually housed and subjected to handling sessions for 3 days. For contextual fear conditioning, mice were placed in a novel rectangular chamber (25 × 33 × 28 cm) with white acrylic side walls, transparent plastic top, front, and rear walls, and a stainless steel grid floor (0.2 cm diameter, spaced 0.5 cm apart) (O'Hara & Co., Ltd) in a sound-proof room with no specific scent. Light-emitting diode (LED) lights, attached to the ceiling above the apparatus, illuminated at 100 lux. Three foot shocks (2 s, 0.75 mA) were administered at timepoints of 208, 298, and 388 s after the animals were placed in the chamber. Mice were returned to their home cage 60 s after the final shock.

For contextual fear memory extinction training, mice were subjected to 2 trials of context re-exposure with a 120 min interval. For each trial, mice were placed in the chamber where fear conditioning was conducted for 30 min without foot shock presentation. For the memory test on days 2 and 3, mice were returned to the chamber for 30 and 3 min, respectively, to assess their contextual fear memory recall as measured by their freezing behavior.

Freezing was scored and analyzed automatically with a CCD camera-based system, TimeFZ4 (O'Hara & Co., Ltd). Images were recorded from the top of each chamber using the camera (two frames per second). For the analysis of images, the gap area (pixel) between the contour of mice in one frame and that in the next frame was identified. If the gap area was under 20 pixels (approximately 30 mm^2) for 2 continuous seconds, mice were judged to have shown freezing behavior. Freezing scores were expressed as the ratio of the freezing period to the experimental period. Two out of 16 wild-type mice whose conditioned responses were not extinguished (greater than 85% freezing) within the first 3 min of the retrieval test on day 2 were excluded from the analysis in Fig. 4.