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Retrieval-Induced Upregulation of Tet3 in Pyramidal Neurons of the Dorsal Hippocampus Mediates Cocaine-Associated Memory Reconsolidation

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Abstract

Background: Memory retrieval refers to reexposure to information previously encoded and stored in the brain. Following retrieval, a once-consolidated memory destabilizes and undergoes reconsolidation, during which gene expression changes to restabilize memory. Investigating epigenetic regulation during reconsolidation could provide insights into normal memory formation and pathological memory associated with psychiatric disorders.

Methods: We used cocaine-induced conditioned place preference to assess the cocaine-associated memory of mice and used chemogenetic methods to manipulate the activity of the pyramidal neurons in the dorsal hippocampus. We isolated the ribosome-associated transcripts from the excitatory neurons in the dorsal hippocampus by RiboTag purification to identify the potential epigenetic regulators, and we specifically knocked down gene expression in pyramidal neurons with a Cre-dependent lentivirus.

Results: Chemogenetically silencing the activity of the pyramidal neurons in the dorsal hippocampus immediately after memory retrieval markedly impaired memory reconsolidation, and the ribosome-associated mRNA level of the ten-eleven translocation (Tet) family methylcytosine dioxygenase Tet3, but not Tet1 or Tet2, was dramatically upregulated 10 minutes after memory retrieval. The protein level of Tet3 in the dorsal hippocampus but not in the anterior cingulate cortex was dramatically increased 1 hour after memory retrieval. Specifically, knockdown of Tet3 in pyramidal neurons in the dorsal hippocampus decreased the activation of pyramidal neurons and impaired the reconsolidation of cocaine-associated memory.

Conclusions: Our findings highlight the new function of the DNA demethylation regulator Tet3 in pyramidal neurons of the dorsal hippocampus in regulating the reconsolidation of cocaine-associated memory.

Keywords: reconsolidation, dorsal hippocampus, CamkIIα^+ neuron, Tet3, cocaine-associated memory

Introduction

The formation of long-term memory involves a series of molecular and signaling changes, including gene transcription, protein synthesis, and neuronal plasticity dynamics (Flavell et al., 2013). These changes may occur during learning and can

Significance Statement

Immediately after memory retrieval, a once-consolidated memory destabilizes and undergoes reconsolidation, during which gene expression changes to restabilize memory. Investigating the epigenetic regulators that act during reconsolidation could provide insights into normal memory formation and pathological memory associated with psychiatric disorders. We found that the activity of the excitatory pyramidal neurons in the dorsal hippocampus (dHC) was required for cocaine-associated memory reconsolidation. After memory retrieval, the number of 5hmC+ pyramidal neurons and the ribosome-associated transcripts of the ten-eleven translocation (Tet) family methylcytosine dioxygenase Tet3, but not Tet1 or Tet2, were upregulated in the dHC. The protein level of Tet3 in the dHC was also increased after memory retrieval. Specifically, knockdown of Tet3 in pyramidal neurons of the dHC impaired the activation of pyramidal neurons and memory reconsolidation. Our findings indicate the importance of DNA demethylation of dHC in regulating reward memory reconsolidation and reveal Tet3 as a new regulator of reward memory.

be subsequently retained for a long time. Memory reconsolidation is the process in which previously consolidated memories, having been reactivated, are stored again to maintain, strengthen, or modify existing memories (Haubrich and Nader, 2016). Following retrieval, once-consolidated memory destabilizes and requires gene expression to be restabilized (Jarome and Lubin, 2014). The result of retrieval is a cascade of events that ultimately leads to changes in neuronal plasticity, which mediate long-term memory updating (Alberini and Kandel, 2014). Thus, understanding the molecular mechanisms of gene transcription regulation during the reconsolidation processes could provide crucial insights into normal memory formation and pathological memory associated with psychiatric disorders, such as PTSD and addiction (Jarome and Lubin, 2014).

Memory retrieval induces de novo transcription and translation, which are orchestrated by epigenetic modifications and are pivotal for memory maintenance and modification (Alberini and Kandel, 2014). Epigenetic mechanisms have been widely implicated in synaptic plasticity underlying memory formation (Day and Sweatt, 2010; Sultan and Day, 2011; Zovkic et al., 2013). DNA methylation and histone modifications have emerged as critical transcriptional regulators of gene expression during initial memory encoding (Jarome and Lubin, 2014), whereas studies on epigenetic regulation in memory reconsolidation are few and mainly focused on histone acetylation (Bredy and Barad, 2008; Maddox et al., 2013; Graff et al., 2014). Recent studies indicate that DNA methylation is involved in the regulation of genomic responses during reconsolidation (Flavell et al., 2013; Oliveira, 2016). By contrast, it remains unclear how memory retrieval triggers epigenetic changes, especially demethylation changes, during the reconsolidation phases to impact behavior, and new epigenetic regulators of gene transcription during memory reconsolidation remain to be identified.

Tet3 belongs to the ten-eleven translocation family of methylcytosine dioxygenases, and it promotes DNA demethylation (Langemeijer et al., 2009; Gu et al., 2011; Shen et al., 2014). Despite the abundance of Tet proteins in the brain, little is known about the dynamic changes and the functions of Tet enzymes after memory retrieval. In this study, by using a cocaine-induced conditioned place preference (CPP) paradigm in mice, we found that the activity of pyramidal neurons in the dorsal hippocampus (dHC) after memory retrieval was critical for reconsolidation. The results of RiboTag purification suggested that Tet3 transcripts were upregulated in CamkIIα+ neurons in the dHC after memory retrieval, and the total protein level of Tet3 in the dHC was elevated synchronously. Furthermore, knockdown of Tet3 in pyramidal neurons in the dHC impaired the activation of these neurons and memory reconsolidation. Our findings lead to the hypothesis that Tet3 in pyramidal neurons in the dHC plays a pivotal role in reward memory reconsolidation and that

Tet3 may represent a potential treatment target for memory-related disorders, including drug addiction.

METHODS

Animals and Housing

CamkIIα-Cre (#005359 B6.Cg-Tg(Camk2a-cre)$^{T29-1Stl/J}$), *RPL22-HA* (#011029-B6N.129-Rpl22$^{tm1.1Psam/J}$) mice were purchased from the Jackson Laboratory. All mice were bred onto a C57BL/6J genetic background. We generated the *CamkIIα-Cre::RPL22-HA (+/-)* mice by crossing *CamkIIα-Cre* mice with *RPL22-HA* mice. The 8- to 12-week-old male offspring were used in the experiments (see Figure 3a for a breeding scheme). Genotypes were determined by polymerase chain reaction (PCR) of mouse tail DNA samples. Mice were housed in groups on a 12-hour-light/-dark cycle with food and water available ad libitum. All animal treatment was in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Animal Care and Use Committee of the School of Basic Medical Sciences of Fudan University.

Stereotaxic Surgery

Mice were anesthetized with choral hydrate (40 mg/kg, i.p.) and placed in a stereotactic instrument. Guide cannulae (Plastics One) were bilaterally implanted in the brain and affixed to the skull with dental cement. The intended stereotactic coordinates were as follows: dorsal hippocampus – anterior-posterior (AP) -1.5 mm; medial-lateral (ML) ±1.5 mm; dorsal-ventral (DV) -1.5 mm; ACC – AP +0.5 mm; ML ±0.5 mm; DV -1.2 mm. All mice were given at least 10 days to recover before behavioral experiments. Anisomycin (A601115-0005, BBI) was dissolved at 125 µg/µL as previously described (Nader et al., 2000). Each mouse received a microinjection of 0.5 µL of anisomycin or vehicle through the cannula (0.2 µL/min) immediately after retrieval.

Cocaine-Induced CPP

CPP induced by cocaine hydrochloride (Qinghai Pharmaceutical Firm) was performed by an investigator blinded to the assigned treatments using a 2-chamber (15 cm × 15 cm × 20 cm) apparatus with distinct tactile environments to maximize contextual differences; a manual guillotine door (15 × 20 cm) separated the 2 chambers. On Day 1, mice were allowed to freely explore the entire apparatus for 15 minutes (pretest). The mice that stayed in one chamber for more than 10 minutes were excluded from the experiment. On Days 2, 3, and 4, mice were given an i.p. injection of cocaine (10 mg/kg) and confined to one of the chambers (drug-paired) for 30 minutes; 6 hours later, they received an i.p.

injection of saline (an equivalent volume to that of cocaine) and were confined to the other chamber for 30 minutes (conditioning). On Day 5, mice were allowed to freely explore the entire apparatus for 5 minutes (retrieval) and were randomized into 2 groups, vehicle and Ani treatment, by tossing a coin. On Day 6, mice were allowed to freely explore the entire apparatus for 15 minutes (test) to evaluate CPP scores. The CPP score (sec/min) was defined as the time spent in the cocaine-paired chamber minus the time spent in the saline-paired chamber per minute. After the experiments, all mice used in the cocaine-induced CPP were sacrificed to verify virus expression and cannula positioning.

Virus Infection

The pSico vector (Addgene plasmid no. 11578) (Ventura et al., 2004) allows for stable, Cre-dependent expression of short hairpin RNAs (shRNAs) in cells and transgenic mice (Ventura et al., 2004). shRNAs encoding oligonucleotides (5'-GCTCCAACGAGAAGCTATTTG-3') targeting Tet3 (Ito et al., 2010) were subcloned into the pSico vector using *Xhol/HpaI* restriction sites. *Lenti-Dio-Tet3 shRNA-EGFP* lentivirus was packaged by the Genechem Technology Co., Ltd., and the titer was $5E^{+8}$ TU/mL. AAV-*CamkIIα-Cre-EGFP* ($5E^{+12}$ V.G./mL), AAV-*CamkIIα-mCherry* ($5E^{+12}$ V.G./mL), AAV-*CamkIIα-hM4D(i)-mCherry* ($5E^{+12}$ V.G./mL), and AAV-*RAM-d2tTA::TRE-FLEX-tdTomato* ($5E^{+12}$ V.G./mL) (Addgene plasmid no. 84468) (Sorensen et al., 2016) were packaged by the Obio Technology Co, Ltd. Microinjections were performed using 33-gauge injection needles connected to a 10-μL Hamilton syringe. Each brain nucleus was injected with 0.2 μL of AAV combined with 1 μL of Lenti viruses at a slow injection rate (0.2 μL/min). The intended stereotactic coordinates were as follows: dorsal hippocampus –AP -1.5 mm; ML±1.5 mm; DV -1.6 mm; ACC–AP+0.5 mm; ML±0.5 mm; DV -1.4 mm. All mice were given 2 to 3 weeks to recover before behavioral experiments, and the knockdown efficiency of the virus was checked by quantitative real time polymerase chain reaction (qRT-PCR).

Immunohistochemistry

CamkIIα-cre::Rpl22-HA mice or virus-infected C57BL/6N mice were anesthetized with choral hydrate (400 mg/kg, i.p.), then perfused transcardially with ice-cold saline and 4% paraformaldehyde (dissolved in phosphate buffer [PB]). The brains were removed and postfixed with 4% PBS-paraformaldehyde solution overnight. Then the brains were subjected to dehydration in 20% sucrose solution for 24 hours and 30% sucrose solution for 48 hours at 4°C before being sliced into 30-μm coronal sections. Slices were washed in PBS and then incubated in primary antibody in blocking buffer (10% donkey serum in PBS containing 0.3% Triton X-100) overnight at 4°C. The slices were washed and incubated in secondary antibody for 1 hour and 4′,6-diamidino-2-phenylindole (DAPI) for 5 minutes at room temperature. After being washed in PBS, the slices were mounted in antiquenching mounting medium. For 5-hydroxymethylcytosine (5hmC) staining, permeabilized cells were denatured as previously reported (Ito et al., 2010). The following primary antibodies were used: anti-5hmC (4000, Active Motif, 1:500), anti-EAAC1 (AB1520, Millipore, 1:500), anti-GABA (A2052, Sigma, 1:500), anti-parvalbumin (AgPV100abs, Swant, 1:500), and anti-hemagglutinin (HA) antibodies (H6908-.5mL, Sigma, 1:1000). DAPI (D9564, Sigma, 1:100) was applied as a counterstain. The following secondary antibodies were used: anti-mouse 488 (715-545-150, Jackson ImmunoResearch, 1:500) and anti-rabbit 488 (711-545-152, Jackson ImmunoResearch, 1:500). Images were acquired using a Nikon-A1 confocal microscope with a 20× objective.

Electrophysiology

Coronal sections (300 μm) containing the dHC were cut from mice injected with *AAV-CamkIIα-hM4D(i)-mCherry* or *AAV-CamkIIα-mCherry*, and the slices were prepared as previously described (Zhao et al., 2011). Briefly, the mice were anesthetized with isoflurane and then transcardially perfused with cold protective artificial cerebrospinal fluid (92 mM N-methyl-D-glucamine, 2.5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 30 mM NaHCO$_3$, 20 mM HEPES, 25 mM D-glucose, 2 mM thiourea, 5 mM Na ascorbate, 3 mM Na pyruvate, 0.5 mM CaCl$_2$, and 10 mM MgCl$_2$). After recovering at 32°C to 34°C for 10 minutes, the slices were transferred into a holding chamber containing room-temperature carbogenated artificial cerebrospinal fluid (119 mM NaCl, 2.5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 26 mM NaHCO$_3$, 12.5 mM glucose, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 2 mM thiourea, 5 mM Na ascorbate, and 3 mM Na pyruvate) and stored for 45 minutes prior to recording. The slices were used within 6 hours after preparation.

Whole-cell current-clamp recordings were performed from mCherry-positive and hM4D(i)-mCherry-positive neurons in the dHC with an EPC-10 amplifier and Patchmaster software (HEKA Elektronik). The intracellular solution composition was as follows: 126 mM K-gluconate, 4 mM KCl, 10 mM HEPES, 4 mM ATP-Mg, 0.3 mM GTP-Na$_2$, and 10 mM creatine phosphate (pH 7.2, 290–300 mOsm). The pipette resistance was in the range of 8 to 10 Ω. Current-clamp recordings were filtered at 2.9 kHz and sampled at 5 kHz. To confirm the inhibitory effect evoked by clozapine N-oxide (CNO), we injected 60 pA of current to elicit action potential firing. Current-clamp recording was performed before and after bath application of 10 μM CNO.

RiboTag Purification

Purification of ribosome-associated mRNAs was conducted as described previously with slight modification (Sanz et al., 2009). Mice were decapitated, and the brains were removed immediately. The dHCs were dissected by the coronal sections (according to the stereotaxic coordinates from Bregma -1.0 mm to -2.5 mm) within 5 minutes in ice-cold PBS. The tissues were homogenized in 1 mL of supplemented hybridization buffer (25 mM Tris pH 7.0, 25 mM Tris pH 8.0, 12 mM MgCl$_2$, 100 mM KCl, 1% Triton X-100, 1 mM dithiothreitol (DTT), 1× protease inhibitors [04693159001, Roche], 200 units/mL RNase inhibitor [N2112S, Promega], 100 μg/mL cycloheximide [14126, Cayman], and 1 mg/mL heparin) using a tissue grinder (JXFSTPRP-24, Shanghai Jingxin Industrial Development Co., Ltd). The supernatant was incubated with 10 μL of anti-HA antibody (H6908, Sigma) and 100 μL of Dynabeads Protein G (10003D, Novex, Invitrogen) for 12 hours. Purified mRNA was eluted from the Dynabeads using TRIzol LS (Thermo Fisher Scientific Inc) according to the manufacturer's instructions with the inclusion of a DNase digestion step. An Agilent RNA 6000 Pico Kit (5067-1513, Agilent) and an Agilent 2100 bioanalyzer were used to evaluate the quality of purified mRNA. Samples with RIN numbers <7 were discarded.

RNA Extraction, Reverse Transcription, and Quantitative PCR Analyses

Total RNA was extracted from tissues using TRIzol Reagent (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. Reverse transcription was completed using a PrimeScript RT Reagent Kit (RR037A, Takara). The cDNA was subjected to qRT-PCR using SYBR Premix Ex Taq (RR420A, Takara)

and an Eppendorf Mastercycler PCR System (Eppendorf). The primers are listed in supplementary Table 1.

Western Blotting

Brain tissues were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protein inhibitors) and loaded onto Mini-PROTEAN TGX Precast Gels (Bio-Rad). Western blotting was carried out as described before. Rabbit anti-Tet3 and anti-actin antibodies were purchased from GeneTex (GTX121453) and Sigma (A2066, Darmstadt), respectively. IRDye 700CW-conjugated anti-rabbit antibody was purchased from Rockland Biosciences. The infrared fluorescence images were obtained and quantified with an Odyssey infrared imaging system (Li-Cor Bioscience).

Statistical Analysis

Eight to 12 mice per group were used for behavioral tests, and 4 to 6 mice per group were used for qRT-PCR and biochemistry studies. Data are presented as the mean ± SEM. The Student's *t* test, 1-way ANOVA, or 2-way ANOVA was used for statistical analysis. Bonferroni posthoc analysis was performed after 1-way or 2-way ANOVA. Significance levels were represented as *$P < .05$, **$P < .01$, and ***$P < .001$.

RESULTS

The Dorsal Hippocampus Is Required for Cocaine-Associated Memory Reconsolidation

C57 mice were given an injection of cocaine (i.p., 10 mg/kg) and trained daily for 3 days to associate the drug with one of the CPP chambers. Twenty-four hours later, mice were reexposed to the CPP chambers (retrieval), and they showed significant preference for the cocaine-paired chamber, indicating that the memory had been retrieved. Immediately after memory retrieval, the protein biosynthesis inhibitor anisomycin (Ani, 150 mg/kg, i.p.) or vehicle was injected i.p., and the mice were tested again 24 hours later for the retention (reconsolidation) of cocaine-CPP memory (Figure 1a). The mice injected with Ani after retrieval showed decreased time spent in the cocaine-paired chamber, indicating impaired reconsolidation (Figure 1b: F_{test} (2, 44) = 114.439, $P < .0001$, $F_{treatment \times test}$ (2, 44) = 3.715, $P = .0323$, $F_{treatment}$ (1, 22) = 9.576, $P = .0053$, Veh vs Ani within test, t = 4.060, $P < .001$). Both ACC and hippocampus play an important role in contextual memory (Einarsson et al., 2015). Furthermore, ACC to hippocampus (CA3 to CA1 region) projection mediates memory retrieval (Rajasethupathy et al., 2015), while their roles in memory reconsolidation are unclear. Bilateral infusion of Ani in the dHC (Figure 1c-d), but not in the ACC (Figure 1e-f), immediately after the memory retrieval significantly inhibited the reconsolidation of cocaine CPP (Figure 1d: $F_{treatment}$ (1, 17) = 6.486, $P = .014$. Veh vs Ani within test, t = 2.999, $P = .004$; Figure 1f: $F_{treatment}$ (1, 14) = 0.606, $P = .449$), indicating that the retrieval-induced synthesis of new protein in the dHC is necessary for the reconsolidation of cocaine-CPP memory.

The Postretrieval Activity of the Pyramidal Neurons in the dHC Is Required for the Reconsolidation of Cocaine-Associated Memory

More than 90% of the neurons in the dHC are excitatory pyramidal neurons. To test the potential role of the principle neurons in memory reconsolidation, we injected mice with *AAV-CamkIIα-mCherry* or *AAV-CamkIIα-hM4D(i)-mCherry* in the dHC (dorsal CA1, dorsal CA3, and some dorsal regions of DG) (Figure 2a-b). Treatment with CNO, an activator of *hM4D(i)*, repressed the firing frequency of hM4D(i)-mCherry+ neurons (Figure 2c-d), while CNO had no effect on locomotion (supplementary Figure 1), confirming that the chemogenetic system works well. *AAV-CamkIIα-mCherry* or *AAV-CamkIIα-hM4D(i)-mCherry* was highly expressed in dHC, but barely expressed in ventral hippocampus (Figure 2e). Two weeks after the viral infection, the mice were trained to associate a context with cocaine (Figure 2a). During the retrieval session, the 2 groups spent similar amounts of time in the cocaine-paired chamber (Figure 2f, mCherry vs hM4D(i)-mCherry, t = 1.032, $P = .307$). CNO (2 mg/kg, i.p.) was injected immediately after memory retrieval, and the CPP score was tested 24 hours later. The mice infected with *AAV-CamkIIα-hM4D(i)-mCherry* showed decreased CPP scores compared with the control group (Figure 2f, $F_{treatment}$ (1, 16) = 4.937, $P = .041$, hM4D(i)-mCherry vs mCherry, t = 2.39, $P = .021$), indicating that the activity of pyramidal neurons in the dHC was required for cocaine-associated memory reconsolidation.

Exploring the Potential Regulators of Cocaine-Associated Memory Reconsolidation by Isolating Ribosome-Associated Transcripts from the Pyramidal Neurons in the dHC

The above data suggest that retrieval-induced expressional activation of pyramidal neurons in the dHC plays a pivotal role in memory reconsolidation of cocaine-induced CPP. Thus, we used the RiboTag technique to isolate ribosome-associated (actively translated) mRNAs from the CamkIIα+ pyramidal neurons in the dHC. *CamkIIα-Cre::Rpl22-HA* mice were generated by breeding *CamkIIα-Cre* mice with the *RPL22-HA* mouse line (Figure 3a), resulting in expression of HA-tagged ribosomes exclusively in CamkIIα+ neurons. Excitatory neuronal markers were enriched in RiboTag-purified transcripts of *CamkIIα-Cre::Rpl22-HA* mice, while inhibitory neuronal and glial markers were not (supplementary Figure 2), indicating that the RiboTag purified excitatory-neuron transcripts. After cocaine-associated memory was formed (supplementary Figure 3) and retrieved (Figure 3b), ribosome-associated mRNAs in the dorsal hippocampi of *CamkIIα-Cre::Rpl22-HA* mice were isolated (Figure 3c), and the retrieval-induced changes in ribosome-associated mRNAs (mRNAs that are being actively translated), especially those related to neuronal activity, were assessed by qRT-PCR. As shown in Figure 3d to g, memory retrieval led to upregulation of the translational activity of the immediate early genes, such as *Arc*, *Egr1*, *Fos*, and *Npas4* (Figure 3d, $P = .0094$, F(3, 19) = 5.373 for *Arc*; $P < .0001$, F(3, 19) = 15.66 for *Egr1*; $P = .0072$, F(3,19) = 5.763 for *Fos*; $P = .0007$, F(3, 19) = 9.560 for *Npas4*) (Bozon et al., 2003; Ploski et al., 2011; Besnard et al., 2014; Lv et al., 2015; Webb et al., 2017). Translation of mRNAs for neurotransmitters and receptors such as *Vgf*, *Trh*, and *Gpr68* were upregulated following retrieval, while *Scn2b* was not (Figure 3e, $P < .0001$, F(3, 19) = 31.19 for *Vgf*; $P = .0344$, F(3, 19) = 3.683 for *Trh*; $P = .0411$, F(3, 19) = 3.469 for *Gpr68*; $P = .0571$, F(3, 19) = 3.085 for *Scn2b*). Previous studies have shown that the MAPK/ERK, PKA, and NFκB pathways are involved in memory reconsolidation (Kelly et al., 2003; Boccia et al., 2007; Arguello et al., 2014; de la Fuente et al., 2015). However, the ribosome-associated transcripts of several components of these signaling pathways, including *Ikbkg*, *Prkcz*, *Pde7b*, and *Dusp1*, had no significant change after memory retrieval (Figure 3f). It is likely that the activity but not the biosynthesis of those components induced
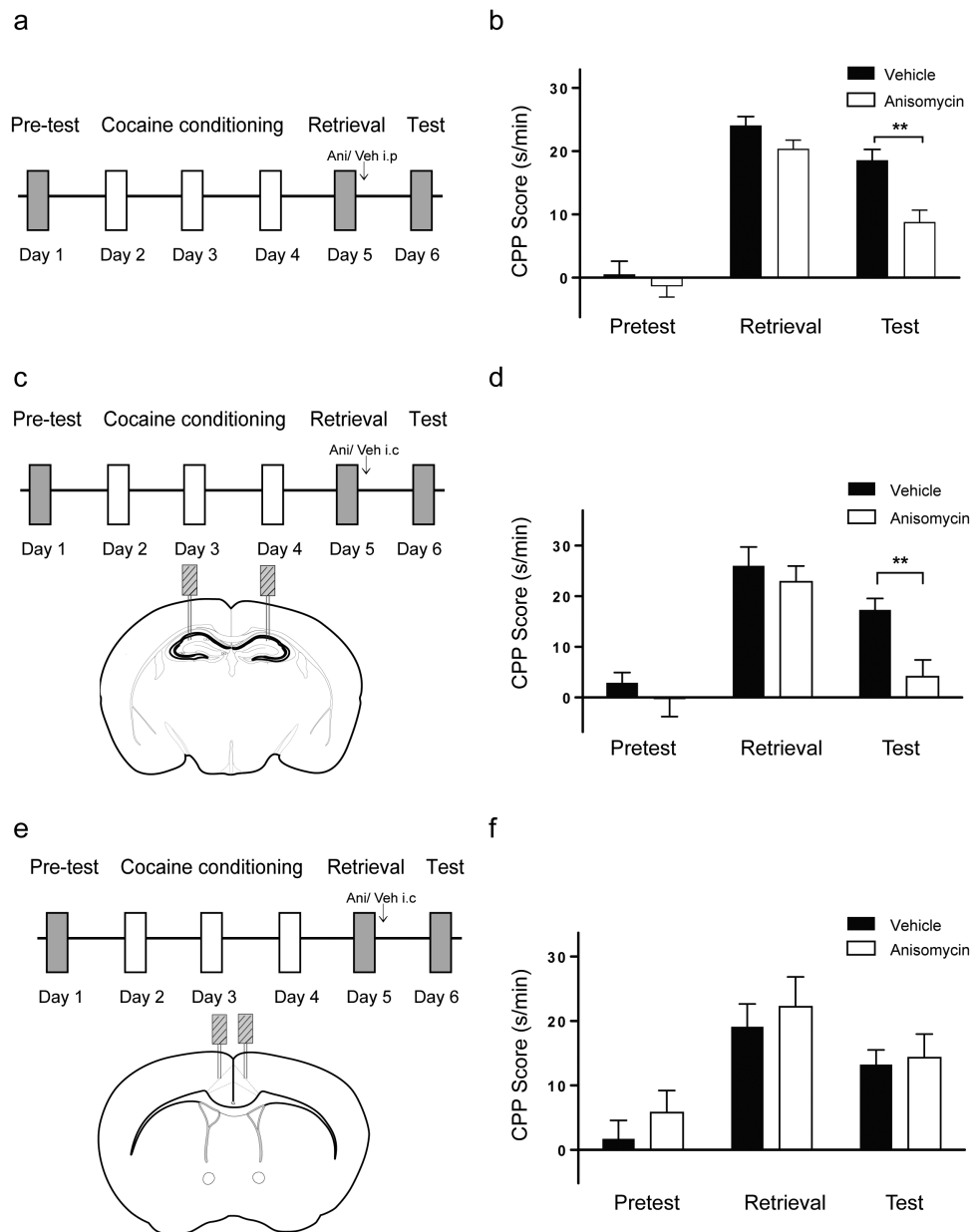
Figure 1. Inhibition of protein synthesis in the dorsal hippocampus disturbs reconsolidation of cocaine-associated memory. (a) Schematic diagram of experimental procedure. Mice were conditioned with cocaine in the conditioned place preference (CPP) apparatus for 3 days. One day after conditioning, mice were reexposed to the CPP apparatus (Retrieval), and the CPP score was determined. Immediately after the retrieval session, mice were given an i.p. injection of vehicle (Veh) or anisomycin (Ani), and CPP was tested 24 hours later (Test 2). (b) An i.p. injection of anisomycin significantly inhibited reconsolidation of cocaine-associated memory (n=11 for vehicle and n=13 for anisomycin). (c,e) Schematic diagram of experimental procedure and cannula placement in the dorsal hippocampus (dHC) or anterior cingulate cortex (ACC). (d,f) Infusion of anisomycin into the dHC (d) but not the ACC (f) immediately after retrieval significantly inhibited cocaine-associated memory reconsolidation. dHC: n=11 for vehicle and n=8 for anisomycin. ACC: n=9 for vehicle and n=7 for anisomycin. **$P<.01$. Veh vs Ani. 2-way ANOVA. Data are presented as the mean ± SEM.

by retrieval is essential for reconsolidation. Accumulating evidence suggests that the epigenetic machinery regulates the formation and stabilization of long-term memory (Sultan and Day, 2011; Cassanelli et al., 2015). Intriguingly, the results of RiboTag purification showed that ribosome-associated transcripts of epigenetic factors, including histone demethylase *Kdm6b*, histone methyltransferase, regulator of active DNA demethylation Gadd45b, and histone deacetylase *HDAC3* in the excitatory neurons were upregulated in CamkIIα⁺ neurons after memory retrieval (Figure 3g, $P=.0045$, $F(3, 19)=6.467$ for *Kdm6b*; $P=.0045$, $F(3, 19)=6.458$ for *Cdyl*; $P=.0021$, $F(3, 19)=7.506$ for *Gadd45b*;

$P=.7460$, $F(3, 19)=0.4129$ for *Hdac3*), indicating the involvement of epigenetic machinery in memory reconsolidation.

Upregulation of Tet3 mRNA and Protein Level in the dHC After Memory Retrieval

Gadd45b plays an important role in active DNA demethylation (Barreto et al., 2007; Ma et al., 2009; Jarome et al., 2015). To explore the possibility that DNA demethylation takes part in memory reconsolidation, we examined the level of 5-hydroxymethylcytosine (5hmC), a demethylation marker (Kaas et al.,
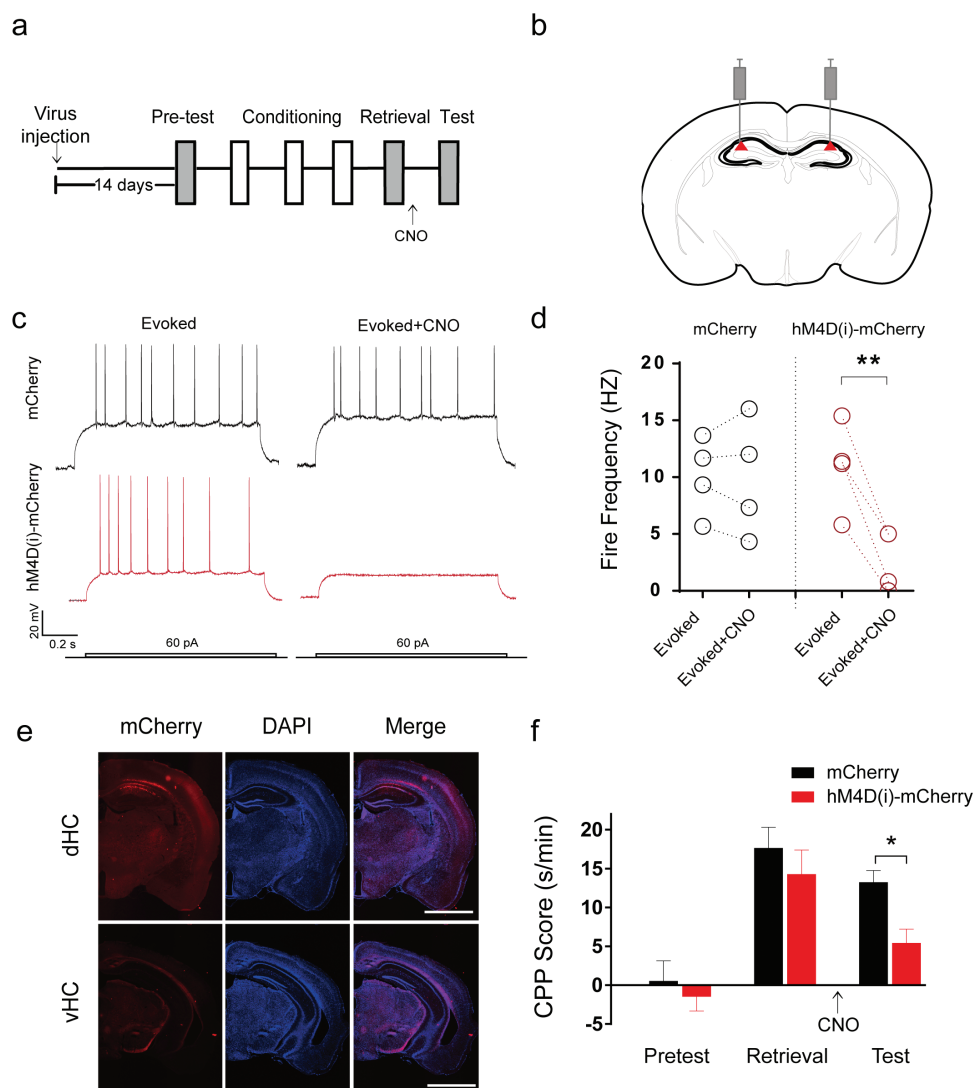
Figure 2. Activity of pyramidal neurons in the dorsal hippocampus is required for reconsolidation of cocaine-associated memory. (a–b) Schematic diagram (a) of chemogenetic inhibition of pyramidal neurons in the dorsal hippocampus (dHC) (b) after retrieval. (c–d) Current-clamp recording of a representative mCherry[+] neuron and a hM4D(i)-mCherry[+] neuron from cocaine-conditioned place preference (CPP)-trained mice. (c) Action potential firing before and after application of 10 μM CNO. Scale bar, 0.2 s, 20 mV. (d) Quantification of the firing frequency of mCherry^{+} neurons and hM4D(i)-mCherry^{+} neurons. n=4 for each treatment; **$P<.01$ Evoked vs Evoked + CNO. Paired Student's *t* test. (e) Representative images of *AAV-CamkIIα-mCherry*-infected dHC and vHC slices. Viruses were barely expressed in ventral hippocampus. Red, mCherry; blue: DAPI. Scale bar, 2 mm. (f) Chemogenetic inhibition of the activity of pyramidal neurons in the dHC after retrieval impaired the reconsolidation of cocaine CPP. n=8 for mCherry and n=9 for hM4D(i)-mCherry; *$P<.05$ mCherry vs hM4D(i)-mCherry; 2-way ANOVA with Bonferroni's posthoc test. Data are presented as the mean ± SEM.

2013), after memory retrieval. We observed a significant upregulation of 5hmC level in pyramidal neurons of the dHC 4 hours after memory retrieval (Figure 4a-b, $P=.0031$, t=6.377, unpaired Student's *t* test). The ten-eleven translocation family of methylcytosine dioxygenases (including Tet1, Tet2, and Tet3), which promote conversion from 5-methylcytosine to 5hmC, have been proposed to play an important part in DNA demethylation regulation (Tahiliani et al., 2009; Ito et al., 2010; He et al., 2011). We found that although the total mRNA levels of Tet1 and Tet2 in the dHC were not significantly changed, the Tet3 mRNA increased after memory retrieval (Figure 4c, $P=.0013$, F(3, 17)=8.253). Furthermore, ribosome-associated Tet3 mRNA in pyramidal neurons was significantly upregulated after memory retrieval (Figure 4d, $P=.0005$, F(3, 16)=10.22), while ribosome-associated Tet1 mRNA and Tet2 mRNA were not, indicating that Tet3 in pyramidal neurons of the dHC might undergo active translation after memory retrieval. Western-blotting analysis showed that the protein level of Tet3 in the dHC, but not in the ACC, was dramatically increased within 1 hour after

memory retrieval (Figure 4e, $P=.0167$, F(3, 21)=4.275, $P=.0129$ for 0 minutes vs 1 hour; Figure 4f, $P=.9880$, F(3, 12) =0.04154).

Tet3 in the Pyramidal Neurons of the dHC Is Required for Cocaine-Associated Memory Reconsolidation

To examine the role of neuronal Tet3, we specifically knocked down Tet3 in pyramidal neurons by co-infection of *AAV-CamkIIα-Cre-EGFP* and *Lenti-Dio-Tet3-shRNA-EGFP* in the dHC of wild-type mice that had acquired cocaine-induced CPP (Figure 5a-c). Tet3 mRNA was significantly decreased in the dHC after *lenti-Dio-Tet3 shRNA-EGFP* infection (Figure 5d, $P=.00896$, t=6.074). The EGFP[+] fluorescence was colocalized with glutamate transporter EAAC1, a marker of glutamatergic neurons (Lammel et al., 2012), not the interneuron marker PV or the inhibitory neuronal marker GABA, indicating that these neurons in the dHC are glutamatergic neurons (supplementary Figure 4). During the memory retrieval
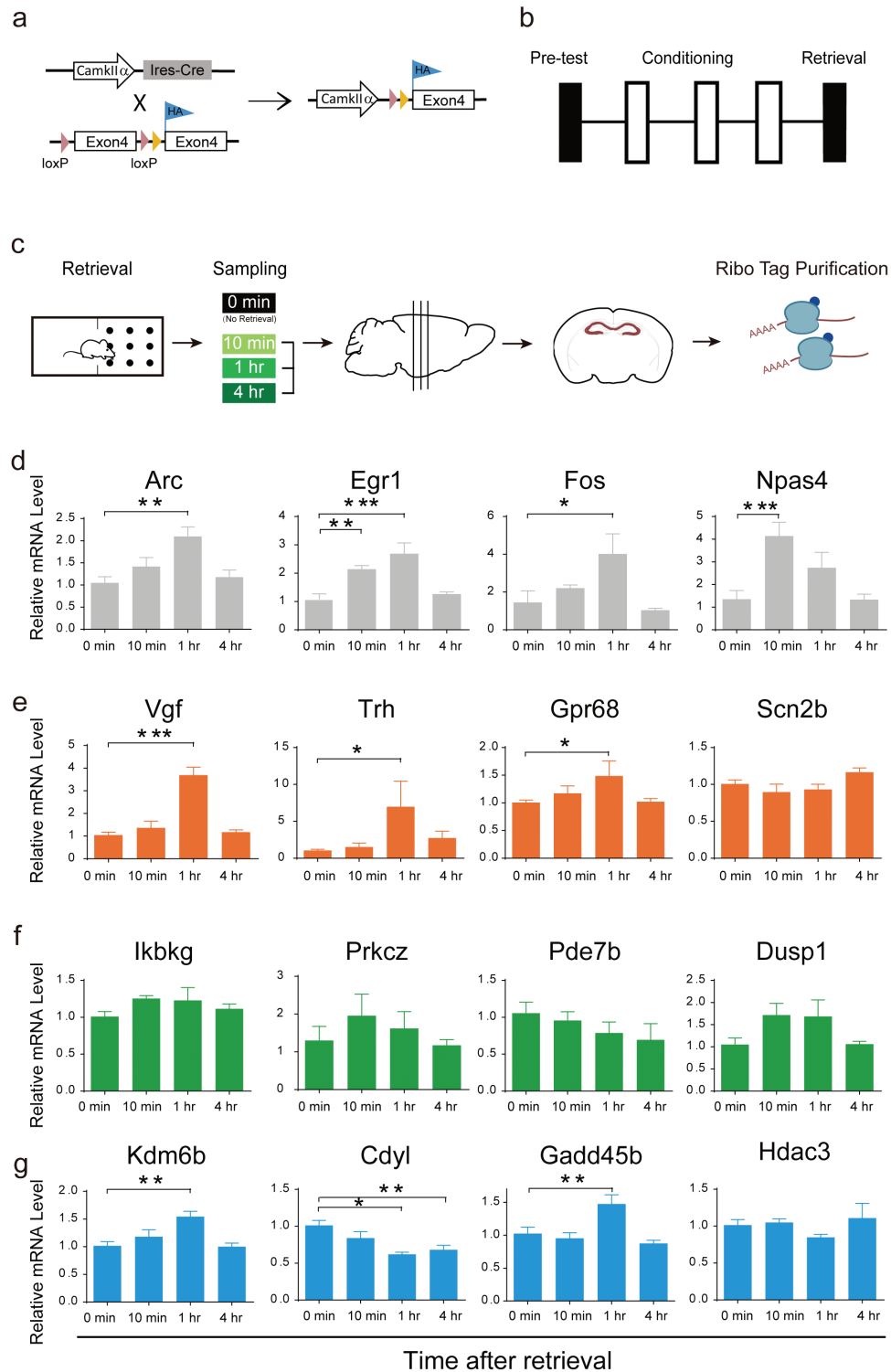
Figure 3. RiboTag purification of ribosome-associated mRNA in pyramidal neurons of the dorsal hippocampus with or without retrieval. (a) Breeding strategy for the *CamkIIα-Cre::RPL22-HA (+/-)* mice. (b) Experimental design for retrieval of cocaine-induced conditioned place preference (CPP) memory. (c) Scheme chart: dorsal hippocampi of *CamkIIα-Cre::Rpl22-HA* mice were separated. The dorsal hippocampi (dHCs) were dissected by the coronal sections (according to the stereotaxic coordinates from Bregma -1.0 mm to -2.5 mm) at 0 minutes (No Retrieval) (n=6), 10 minutes (n=4), 1 hour (n=3), and 4 hours (n=7) after retrieval of cocaine-induced CPP memory. RiboTag purification was conducted using anti-HA antibody and Protein G beads. Ribosome-associated mRNA in CamkIIα⁺ neurons was immunoprecipitated along with HA-tagged ribosomes. Purified mRNA was verified using qRT-PCR. (d–g) qRT-PCR analysis of ribosome-associated mRNAs of immediate early genes (d); neurotransmitters and receptors (e); critical molecules in the MAPK/ERK, PKA, and NFκB pathways (f); and epigenetic regulators (g) in pyramidal neurons at different time points after memory retrieval. ***P<.001, **P<.01, *P<.05. 1-way ANOVA with Bonferroni's posthoc test. Data are presented as the mean ± SEM.
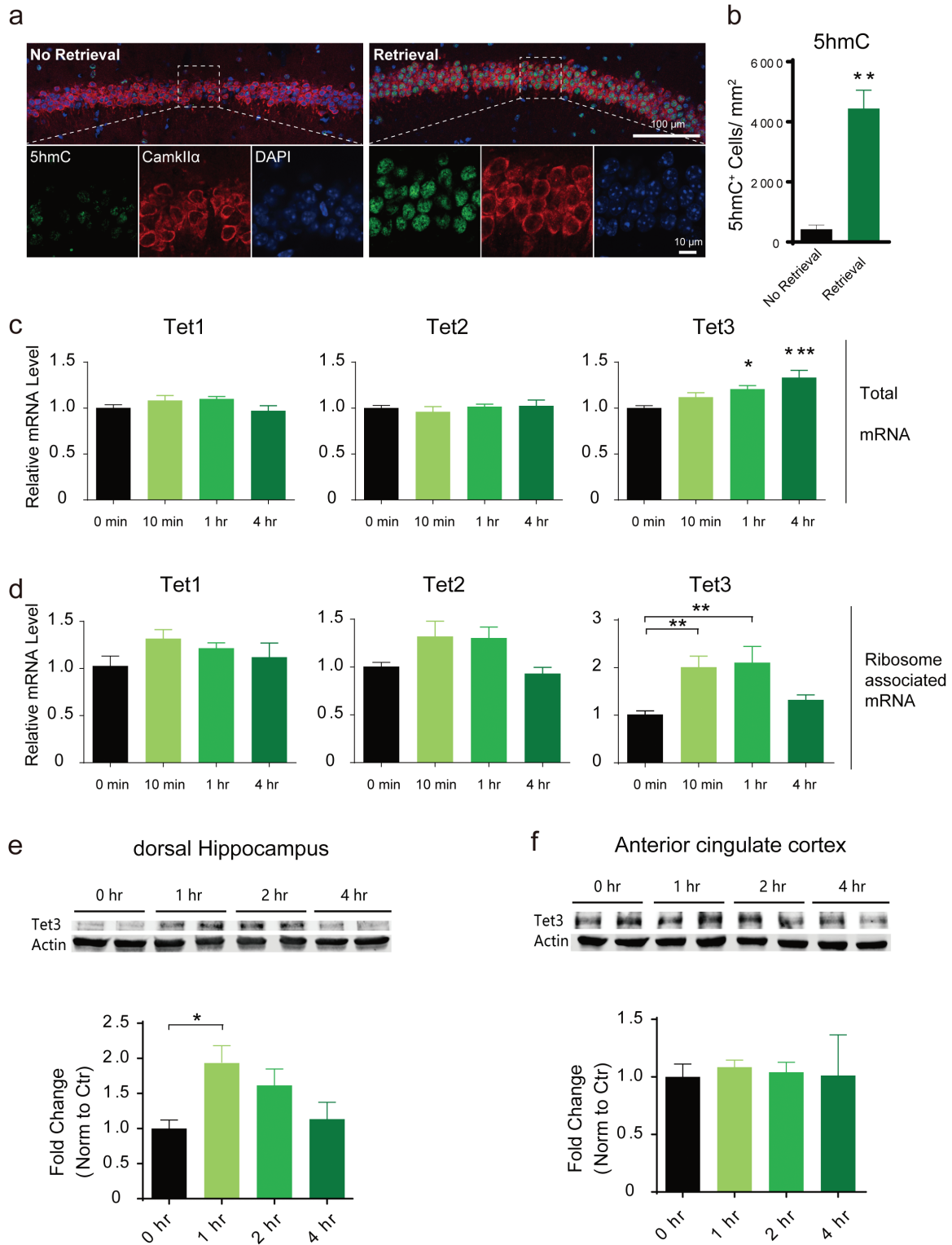
Figure 4. Tet3 in the dorsal hippocampus (dHC) is upregulated after memory retrieval. (a–b) Genomic 5-hydroxymethylcytosine (5hmC) levels are significantly upregulated in the dHC of *CamkIIα-Cre::RPL22-HA* (+/-) mice after memory retrieval. Green, 5hmC; red, CamkIIα; blue, DAPI. Scale bar: upper, 100 µm; lower, 10 µm. n=3 for No Retrieval and n=3 for Retrieval. Data are presented as the mean ± SEM. **P<.01. Unpaired Student's t test. (c–d) qRT-PCR analysis of total (c: n=6, 5, 5, and 5 for each group) or ribosome-associated (d: n=6, 4, 3, and 7 for each group.) mRNA levels of Tet1, Tet2, and Tet3 in CamkIIα+ neurons from dorsal hippocampus at different time points after memory retrieval. *P<.05, **P<.01, ***P<.001 vs 0 hr (No Retrieval) Group. (e–f) Representative images of Western blots and quantification of the total Tet3 protein levels in the dHC (e: n=7, 6, 6, and 6 for each group) and anterior cingulate cortex (ACC) (f: n=4, 3, 3, and 3 for each group) at different time points after memory retrieval. *P<.05 vs 0 hr group. 1-way ANOVA with Bonferroni's posthoc test. Data are presented as the mean ± SEM.
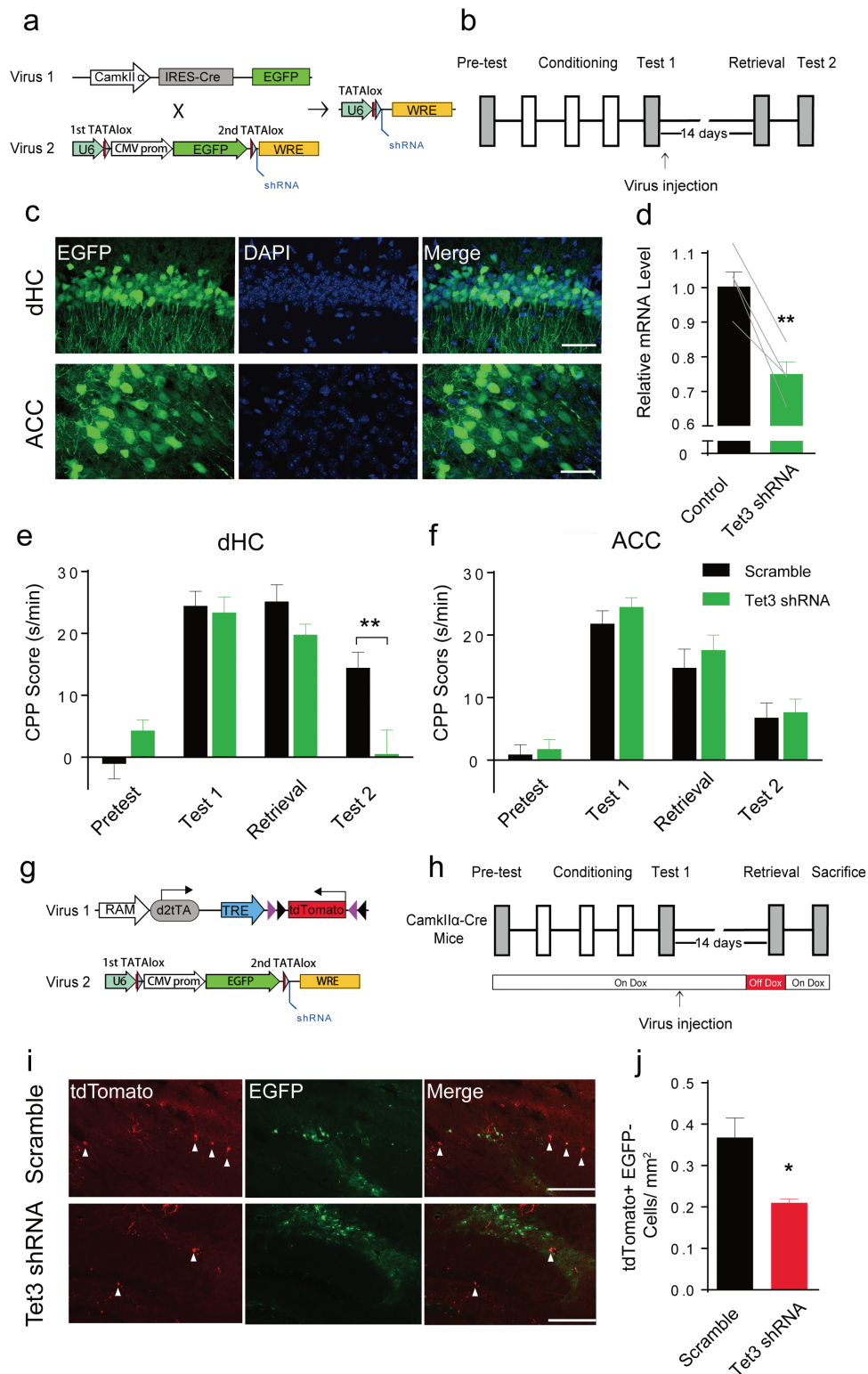
Figure 5. Downregulation of Tet3 in pyramidal neurons of the dorsal hippocampus (dHC) impairs memory reconsolidation. (a) Schema chart. Cre-dependent expression of EGFP/shRNA by a pSico lentivirus in CamkIIα+ neurons. (b) Experimental design. Downregulation of Tet3 in pyramidal neurons of the dHC after memory acquisition. (c) Representative images of virus expression in the dHC and anterior cingulate cortex (ACC). Green, EGFP; blue, DAPI. Scale bar, 40 μm. (d) Verification of Tet3 knockdown efficiency. qRT-PCR of the Tet3 mRNA level in the dHC to verify the knockdown efficiency of the lentivirus *Lenti-Dio-Tet3 shRNA-EGFP*. Paired Student's *t* test, n=4. (e–f) Expression of Tet3 shRNA in pyramidal neurons in the dHC (e: n=13 for scramble and n=9 for Tet3 shRNA), but not in the ACC (f: n=17 for scramble and n=22 for Tet3 shRNA), after memory acquisition inhibited reconsolidation of cocaine-associated memory. **P<.01. 2-way ANOVA with Bonferroni posthoc test. Data are presented as the mean ± SEM. (g–j) Schematic diagram of the viruses (g) and experimental procedure (h) used to label the activated pyramidal neurons in the dHC during memory retrieval. Quantification of the density of tdTomato+EGFP- cells in the dHC. *Lenti-Dio-Tet3 shRNA-EGFP* significantly downregulated the retrieval-induced activation of pyramidal neurons in the dHC (i,j). Red, tdTomato; Green, EGFP; Arrowheads, activated neurons, scale bar: 200 μm. n=4 for scramble and n=4 for Tet3 shRNA. Data are presented as the mean ± SEM. *P<.05. Unpaired Student's *t* test.

session, the CPP score of mice co-injected with *Lenti-Dio-Tet3-shRNA-EGFP* showed no difference compared with the *Scramble shRNA* group, indicating that the ablation of Tet3 in pyramidal neurons does not affect memory retrieval (Figure 5e, F_{virus} (1, 80) = 4.044, P = .0477. Retrieval: scramble vs Tet3 shRNA, P = .6165). However, when assessed 24 hours after memory retrieval, the CPP score of the *Lenti-Dio-Tet3-shRNA-EGFP*-infected group was significantly decreased (Figure 5e, Test 3: scramble vs Tet3 shRNA, P = .0014). In contrast, mice injected with *Lenti-Dio-Tet3-shRNA-EGFP* in the ACC showed no change in CPP score during the retrieval and reconsolidation sessions (Figure 5f, F_{virus} (1, 148) = 1.454, P = .2298). These results identify an important and specific role of neuronal Tet3 in the dHC in regulating cocaine-associated memory reconsolidation. To test whether Tet3 regulates the activity of pyramidal neurons in the dHC, we co-injected *AAV-RAM-d2tTA::TRE-FLEX-tdTomato* (Sorensen et al., 2016) and *Lenti-Dio-Tet3-shRNA-GFP* into the dHC of CamkIIα-Cre mice after cocaine conditioning, then subjected these mice to retrieval while off Dox (Figure 5g-h). Results showed that after memory retrieval, the density of tdTomato⁺EGFP⁻ cells in the dHC of *Lenti-Dio-Tet3-shRNA-EGFP*-infected group was significant downregulated (Figure 5i-j, P = .0169, t = 3.276), indicating that Tet3 regulates the activity of pyramidal neurons in the dHC.

Discussion

Our study explored the critical molecules, cell type, and nuclei required for cocaine-associated memory reconsolidation. We found that activity of excitatory pyramidal neurons in the dorsal hippocampus is responsible for cocaine-associated memory reconsolidation. We also showed that memory retrieval led to enhanced expression of Tet3, an important epigenetic modulator mediating DNA demethylation. Knockdown of Tet3 in CamkIIα⁺ neurons of the dHC impaired the activation of pyramidal neurons and cocaine-associated memory reconsolidation, indicating that the upregulation of Tet3 is pivotal for memory reconsolidation.

Given that memory reconsolidation is sensitive to protein synthesis inhibitors (Nader et al., 2000), it is reasonable to speculate that critical molecules mediating memory reconsolidation are among the proteins synthesized de novo upon memory retrieval. However, the study of cellular and molecular dynamics after memory retrieval is difficult: proteomics lacks cell specificity, while laser-capture microdissection and fluorescence-associated cell sorting lack information on protein synthesis (Shin et al., 2014). By dissecting target brain nuclei and applying RiboTag purification (Heiman et al., 2008; Sanz et al., 2009), we were able to circumvent previous limitations and capture ribosome-associated transcripts, which might be a partial, cell type-specific representation of the proteins being synthesized. Based on our findings, ribosome-associated transcripts of immediate early genes, ligands, and receptors were enhanced after memory retrieval, which is in line with previous reports (Bozon et al., 2003; Lv et al., 2015). However, no differences were detected in the molecules related to the MAPK/ERK pathway, PKA pathway, and NFκB pathway, which are involved in memory reconsolidation (Kelly et al., 2003; Boccia et al., 2007; Arguello et al., 2014). One explanation for this is that these signaling pathways might rely not only on translational activity but also, and to a greater extent, on protein modification and interaction activity. Accordingly, we confirmed the ribosome-associated transcripts of several well-known molecules involved in reconsolidation were enhanced using RiboTag purification, indicating that our system is reliable.

Epigenetic regulation plays a critical role in preserving long-term changes in neuronal cells. Maddox and colleagues (Maddox et al., 2014) discovered that inhibiting DNA methyltransferase (DNMT) impairs fear memory reconsolidation. DNA demethylation is typically thought to positively regulate gene transcription by promoting the binding of transcription factors (Levenson and Sweatt, 2005; Levenson et al., 2006; Miller et al., 2010; Jarome and Lubin, 2014), and we discovered that Tet3, which promotes DNA demethylation, is critical for reward-related memory reconsolidation. It seems probable that both DNA demethylation and methylation are required for memory updating and supported the notion that the dynamic procedures of epigenetic modification, such as DNA demethylation and methylation, are important for the formation of long-term memory. Our findings led to the hypothesis that DNA demethylation might take part in promoting the transcription of synaptic plasticity-related genes during memory reconsolidation.

The dynamic changes in Tet3 methylcytosine dioxygenases after memory retrieval may be involved in retrieval-dependent memory updating. Our findings raise the questions of what is the downstream target of Tet3 and how Tet3 regulates downstream genes. Future studies aimed at target genes of Tet3 may contribute significantly to our understanding of the fundamental mechanism of the epigenetic machinery underlying memory. Neuronal activation regulates the dynamics of the status of chromatin in a precisely timed manner, with subsequent alterations in the gene expression profile. The identification of the components of different signaling pathways involved in memory-related epigenetic regulation will provide mechanistic insights into the formation of long-term memory.

Of note is that all the memory experiments in this work were conducted with the cocaine-induced CPP, which is a reward-associated memory model. Further studies using other memory paradigms such as auditory fear conditioning and inhibitory avoidance would help us better confirm the critical function of Tet3 in memory reconsolidation. In conclusion, we demonstrate for the first time, to our knowledge, that Tet3 of pyramidal neurons in the dHC mediates cocaine-associated memory reconsolidation by acting as an epigenetic modulator. In particular, our study illustrates that Tet3 may represent an exciting potential target for the therapy of psychiatric disorders.

Acknowledgments

Statement of Interest

The authors declare no competing financial interests.

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