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Hippocampus and Prefrontal Cortex Modulation of Contextual Fear Memory Is Dissociated by Inhibiting De Novo Transcription During Late Consolidation

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Abstract

To uncover the factors that dictate the persistence of a memory, it is critical to determine the molecular basis of consolidation. Here, we submitted male adult C57/BL6 mice to contextual fear conditioning using 1US (US: foot-shock, 0.7 mA, 2 s) or 5US, to generate recent (24 to 48 h) and remote (30 days) memories, respectively. To access the functional role of de novo transcription, we injected actinomycin D (ActD: 2.5 ng/side) directly into the dorsal hippocampus (HIP) or dorsomedial prefrontal cortex (dmPFC), 0 (early consolidation) or 12 h (late consolidation) after training. Our results showed that de novo transcription at 0 h was required for recent and remote memories. However, 12 h was a critical time point to memory persistence. In the dHIP, de novo transcription at 12 h post-training differentiated the recent memory from the remote. In the dmPFC, ActD affected memory formation depending on the training intensity (1 or 5US). Specifically, freezing was amplified after 5US conditioning. Furthermore, inhibiting de novo transcription at 12 h post-training in the dmPFC rapidly increased c-Fos expression in the amygdala. Altogether, our results indicate that contextual fear memory duration is particularly sensitive to de novo transcription in the dHIP and dmPFC, at a specific time point of late consolidation.

Keywords Contextual fear memory · Late consolidation · Dorsal hippocampus · Dorsomedial prefrontal cortex · De novo transcription

Introduction

Since it was named [1], the concept of memory consolidation has undergone adjustments, as a result of the massive production of data by the field of learning and memory [2]. Nevertheless, the temporal dynamic underlying the consolidation theory still prevails.

Consolidation initiates immediately after acquisition. At the cellular level, a set of molecular events occurs within few hours to modulate synaptic strength [3–5] and stabilize the memory [6, 7]. This period is named synaptic or early consolidation [8]. For the memories that last longer, it is assumed that additional consolidation occurs [9–11] and, in this case, is named systems or late consolidation [2, 8]. The temporal boundary between synaptic and system consolidation is still under investigation [12–16], though it is likely that both occur simultaneously, at least in a particular time window.

Several studies have unraveled early consolidation mechanisms using the fear conditioning paradigm [17–27]. A time window of up to 3 h has been shown to be sensitive to amnesic agents [18, 19, 22, 28, 29]. For instance, time-specific peaks of protein synthesis are observed in the dorsal hippocampus during early consolidation of contextual fear memory [17, 27]. Additionally, the expression of immediate-early genes (IEG), such as c-Fos and Arc, appears to be markedly related to synaptic plasticity and memory formation [30–32].

In contrast, little is known about the mechanisms involved in late consolidation. Inhibiting protein synthesis or depleting BDNF and Arc with antisense oligodeoxynucleotide in the hippocampus, 12 h after learning, impairs remote fear memory without affecting recent memory [16, 33]. At the same time

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point after contextual fear learning, sharp-wave ripples and c-Fos expression increased in the hippocampus [32], while Arc expression increased in the basolateral amygdala (BLA) [34]. Furthermore, blocking dopamine D1 receptors 12 h after training in inhibitory avoidance task impaired remote, but not recent memory in rats [14].

Despite evidence supporting the relevance of 12 h to memory duration [15, 16, 32–34], most reports about remote memory disregard specific consolidation time points. For instance, pre-training blockade of hippocampal CA3-CA1 communication compromised recent memory in CA3-TeTx mice, while the same manipulation, though applied post-training, impaired only the remote memory [35]. Nevertheless, the genetic manipulation that blunted CA3-CA1 circuits occurred in a long-time frame (weeks). In another study, the optogenetic inhibition of the entorhinal cortex inputs to prefrontal cortex impaired remote memory (22 days), without affecting recent memory (2 days). However, this inhibition occurred during the entire training session [36].

Here, we focus at specific time points of consolidation and evaluated whether de novo transcription during early and late consolidation modulates recent and remote memory in dHIP and dmPFC. By varying the amount of unconditioned stimulus (US) in contextual fear conditioning, we were able to separate recent from remote memory at the behavioral level. Our results suggest that de novo transcription in dmPFC and dHIP, immediately after training, is essential for recent and remote memory, regardless of the training intensity (1US versus 5US). However, hippocampal de novo transcription 12 h post-training is necessary for remote, but not recent memory consolidation. Interestingly, inhibiting de novo transcription in the dmPFC, 12 h after 5US, amplified conditioned freezing and also increased c-Fos expression in the amygdala during late consolidation. Altogether, our results show for the first time that contextual fear memory is differentially affected by de novo transcription in dHIP and dmPFC, during late consolidation.

Material and Methods

Animals

Male C57/BL6 mice (8- to 10-week-old) were housed in groups of three to five per cage in a temperature-controlled room (22 ± 2 °C) with a 12:12 light–dark cycles. Food and water were provided ad libitum. All experimental procedures were approved by the Animal Use Ethics Committee of Universidade Federal de Minas Gerais (322/2014) and carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Contextual Fear Conditioning

Mice were trained and tested in a conditioning chamber (390 × 470 × 320 mm; Insight Ltda, Brazil) with a grid floor by which the foot-shock (US) was delivered (0.7 mA, 2-s scrambled foot-shock). Training session started with a 120-s habituation to the context. Animals from the control group (US^-) returned to their home cage immediately after habituation. Animals from 1US and 5US groups received 1- or 5-foot-shocks, respectively. In 5US group, shocks were delivered with a 60s interval between them. After 1US or the last of 5US, animals remained in the conditioning chamber for additional 30 s.

Recall test session consisted of placing the animal in the conditioning chamber for 300 s. Freezing behavior (defined as complete lack of movement, except for respiratory movements) was quantified every 5 s during the entire testing session. Contextual fear memory was represented as the percentage of time during which mice spent freezing. Same animals were tested for recent and remote memories, 2 and 30 days after training, respectively. Extinction protocol consisted in testing mice, as mentioned before, for 4 consecutive days.

To evaluate generalization, a different set of mice were trained and tested as described before, except that test sessions were performed in two distinct contexts: the conditioned and the non-conditioned one. Contexts differed by the wall's color (black or striped), the grid floor pattern, and the smell (70% ethanol or 0.01% acetic acid) [23].

Stereotaxic Surgery

Mice received intramuscular injection of tramadol (0.05 mg/kg) and 30 min later were anesthetized with isoflurane (3% induction, 1–2% maintenance) and placed in a stereotaxic apparatus (Digital Stoelting, 51730D, USA). Bregma and lambda were aligned at the same horizontal and vertical planes. Small holes (~0.7 mm) were drilled directed towards the CA1 region of the dorsal hippocampus (from bregma: AP − 1.9; LL ± 1.6; DV − 1.0) or dmPFC (from bregma: angle 14°; AP + 2.4; LL = ± 0.3; DV − 1.8). Bilateral guide cannulae (22G, 7 mm) containing dummy cannulae were inserted and fixed in the skull with zinc cement followed by dental acrylic [37]. Animals were allowed to recover for at least 5 days.

Drugs and Infusion

We used actinomycin D (ActD, Sigma-Aldrich), a potent inhibitor of RNA synthesis [38] to assess the role of de novo RNA synthesis in fear memory consolidation. Mice received ActD (5 ng/μl in 5% DMSO) or vehicle (5% DMSO). Drugs were administered immediately (0 h) or 12 h after fear conditioning.

Mice were gently held to remove the dummy cannulae. An injector cannula (30G, 8 mm) was coupled to the guide cannula at the time of the infusions. A microinfusion pump was used to control infusions through a polyethylene tubing (PE20) connected to a 10 μl syringe (Hamilton). Drugs were infused bilaterally at a rate of 0.5 μl/min, in a volume of 0.5 μl/side. Injector cannula remained in place for 1 min after infusion to avoid drug diffusion into the injection track.

Histology

At the end of the experiments, mice were euthanized and their brains were immediately removed and stored in 4% paraformaldehyde for 1 day, followed by 2 days in 30% sucrose. Coronal sections (100-μm thick collected proximal to cannulae tracts) were cut on a cryostat (− 20 °C). Slides were stained with neutral red and injection sites were verified by light microscope. Only mice with correct cannulae placements were included in statistical analyses (Fig. 1).

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Animals were euthanized and their brains were rapidly removed. Hippocampus and prefrontal cortex were bilaterally dissected on ice. Total RNA from tissues samples were isolated with Trizol® (Invitrogen, Burlington, EUA) following the manufacturer's instructions. RNA was resuspended in 20 μl of RNAse-free water (Ambion®). The concentration was analyzed by Nanodrop (NanoDrop—ThermoScientific, Wilmington, USA) and the quality by gel electrophoresis. To prepare the cDNA, we used 400 ng of total RNA in a reverse transcription reaction with the final volume of 20 μl. Using Power SYBR® Green PCR Master Mix and StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Foster City, USA), quantitative PCR was performed to evaluate mRNA levels of following genes: Arc (NM_018790.), BDNF (NM_001285416.1), and RPL32 (NM_172086.2).

Primers were designed using Primer3plus software: Arc (forward: GCTGAAGCAGCAGACCTGA; reverse: TTCACTGGTATGAATCACTGCTG), BDNF (forward: ATGAAAGAAGTAAACGTCCAC; reverse: CCAGCAGA AAGAGTAGAGGAG), RPL32 (forward: TGACTGGT GCCTGATGAACT; reverse: GCTGCCATCTGTTT TACGG), actin (forward: TGGAATCCTGTGGCATCCAT GA; reverse: AATGCCTGGGTACATGGTGGTA). All RT-qPCRs showed good quality of amplification. Specificity and efficiency of primers were tested and confirmed by serial dilution method. Samples were prepared in triplicate, and actin expression was used as normalizer. We used the Livak and Schimittgen [39] method and the following formula: $2^{-(\Delta Ct\ \text{gene of interest} - \Delta Ct\ \text{actin})}$. All data was shown as fold to control. Our treatment did not affect the Actin CT (data no shown).

c-Fos Expression by Immunohistochemistry

Mice were injected with vehicle (5% DMSO) or ActD into the dorsomedial prefrontal cortex 12 h after fear conditioning. Naïve animals were used as control group. After 90 min, they were anesthetized with ketamine (100 mg/kg) and xilazine (10 mg/kg) and transcardially perfused with 0.01-M phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). Brains were post-fixed overnight in 4% PFA and stored in 30% sucrose (*w*/*v*) solution at 4 °C. Sections (40-μm thick) were cut with a cryostat (Leica, CM 1860)

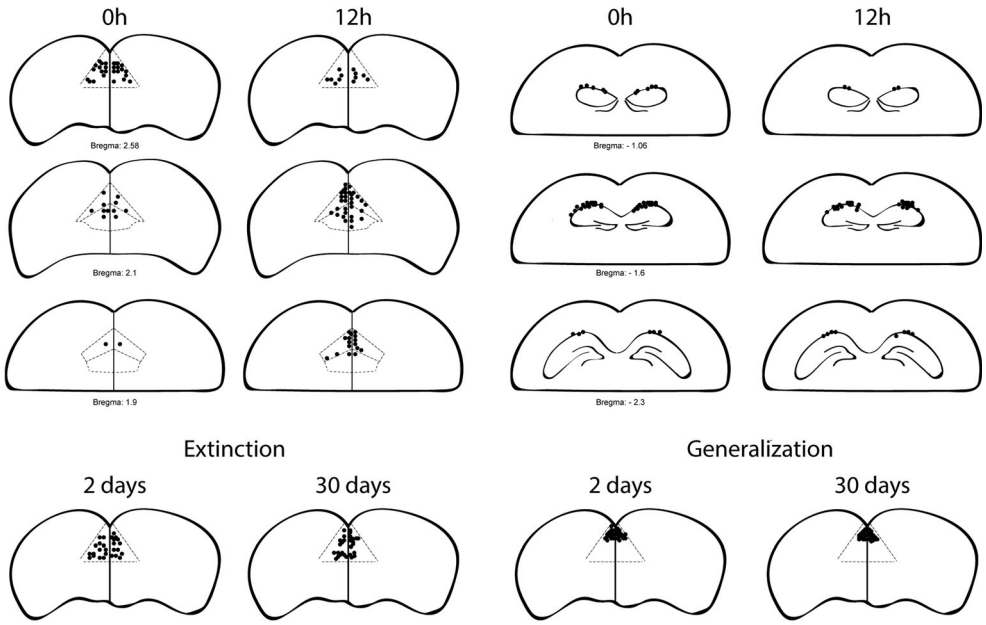

Fig. 1 Schematic drawings of brain slices, emphasizing the target areas: CA1 region of the dorsal hippocampus (bregma − 1.06 to − 2.3) and dorsomedial prefrontal cortex (bregma 2.58 to 1.9). Each black circle corresponds to one cannula placement. All animals used in behavioral analysis are represented

and kept at − 20 °C in solution containing 30% ethylene glycol, 30% sucrose, 1% polyvinylpyrrolidone, and 0.1-M PBS. For free-floating immunohistochemistry, sections were washed in PBS (10 × 6min) and incubated in 0.1-M glycine in PBS for 10 min. Next, they were washed in PBS (3 × 6 min) and treated for another 10 min with 1% H_2O_2 solution in PBS. Sections were washed (5 × 6 min) and then rinsed with PBS containing 0.3% Triton X-100, then washed again in PBS (3 × 6 min). Sections were transferred for a blocking solution (BSA 3% in PBS) for 1 h and then incubated for 48 h at 4 °C with c-Fos antibody 1:1500 (Santa Cruz, sc-52) in the same blocking solution. Sections were washed in PBS (10 × 6 min) and incubated with the secondary antibody (1:1000, biotinylated anti-IgG antibody goat anti-rabbit; Vector Laboratories) for 2 h at room temperature. Subsequently, the sections were washed in PBS (8 × 6 min) and treated with avidin-biotin complex (1:500 in PBS; Vector Laboratories) during 1 h at room temperature. Sections were washed (3 × 6 min) in PBS and in TRIS-HCl (0.05 M, pH 7.6) (3 × 6 min) and incubated in revealing solution (0.2-mg/ml diaminobenzidine (DAB), 25-mg/ml nickel sulfate, and 0.0025% H_2O_2 in TRIS-HCl 0.05 M) for 10 min. Finally, the sections were washed in TRIS-HCl (3 × 6 min) and PBS (3 × 6 min) and mounted in Entellan.

Imaging and Analysis

Slides were observed under a Zeiss Axio Imager.Z2 microscope bright-field function. Sections were photographed with a ×20 objective for quantification and ×5 objective for representative images. The TIFF-format micrographs were analyzed using ImageJ software (http://rsbweb.nih.gov/ij/). We delimited manually the following areas: hippocampus (CA1, CA3, DG), Amy (Ce and BLA), and PAG. Counting for $cFos^+$ cells was performed using the threshold tool to construct a mask separating stained cells from the background (same pixel threshold level was held for all images analyzed). An identified object was only counted as a cell if the overall diameter was within 25 and 90 μm and the circularity index was higher than 0.4. The total number of cells was normalized by area in mm^2.

Statistical Analysis

All analyses were performed using the *Graph Pad Prism 7* statistics software. One-way ANOVA followed by Bonferroni's post hoc test was used in Fig. 3b–e. Two-way ANOVA [factor 1: US (US^-, 1US, or 5US) and factor 2: treatment (vehicle or ActD)] with Bonferroni's post hoc was used in Figs. 3g–i, 4b–i, and 6. We used two-way repeated measures ANOVA in Fig. 2b, c (US versus day) and Fig. 5b–e (day versus drug). For generalization data (Figs. 2e and 5f–g), we performed unpaired *t* test.

Results

Foot-Shocks (US) Number Dictates the Duration of Contextual Fear Memory

To induce fear memories that differ in duration, we used one (1US) or 5 (5US) foot-shocks during the training session of classical conditioning. Afterward, contextual fear memory was tested 24 h (recent memory) and 30 days (remote memory) (Fig. 2a). Compared to control group (US^-), both 1US and 5US were sufficient to induce recent memory, although 5US increased fear behavior compared to 1US. Interestingly, remote memory was only induced by 5US, since animals conditioned to 1US behaved like the control group when tested 30 days after conditioning (interaction: $F_{(2,\ 12)} = 4.8$; $p = 0.02$; test: $F_{(1,\ 12)} = 8.4$; $p = 0.01$; US: $F_{(2,\ 12)} = 43.6$; $p < 0.0001$) (Fig. 2b).

Next, we evaluate the extinction curve to verify whether the higher freezing levels induced by 5US were sensitive to extinction. As expected, 1US group returned to the level of the control group (US^-) faster than the 5US group (Fig. 2c). However, freezing in the 5US group gradually decreased along the re-exposure sessions. At day 50, there was no difference between groups (interaction: $F_{(3,\ 42)} = 4.3$; $p = 0.009$; test: $F_{(3,\ 42)} = 56.9$; $p < 0.0001$; US: $F_{(1,\ 14)} = 16.6$; $p = 0.001$). Our results show that fear memory yielded by 5US is susceptible to extinction.

Context fear memories gradually lose details leading to an increase in context generalization over time [40]. Therefore, we tested generalization of the remote memory. Animals were submitted to CFC, and 30 days after training, they were tested in the context A (conditioned context) or context B (similar context) (Fig. 2d). Animals tested in the similar context (context B) showed noticeable less freezing than animals tested in context A ($t_{(12)} = 3$; $p = 0.009$), suggesting that 5US induces a fear memory that does not generalize 30 days after learning (Fig. 2e).

Hippocampal Gene Expression 12 h After Acquisition Is a Molecular Marker of Contextual Fear Conditioning Induced by 5US

Next, we sought to investigate molecular markers that could differentiate recent and remote memories induced by 1US and 5US, respectively. Previous studies have indicated 12 h post-training as a critical time point for hippocampal molecular changes [15, 16, 33] that underlie the consolidation of remote memory. Thus, we tested whether BDNF and Arc mRNA expression would change in the hippocampus, 12 h after conditioning (Fig. 3a). We observed an increase in BDNF ($F_{(2,\ 14)} = 3.9$; $p = 0.04$) and Arc ($F_{(2,\ 12)} = 7.6$; $p = 0.007$) expression only in the 5US group (Fig. 3b, c).

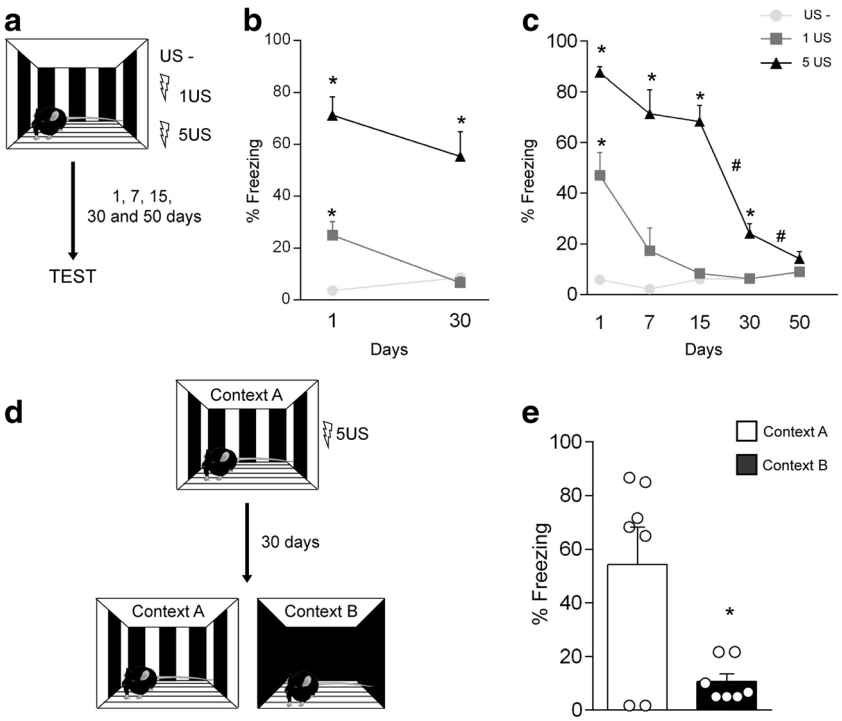

Fig. 2 Contextual fear conditioning using 5US induces remote memory that last 30 days, but can be extinguished and does not generalize. **a** Animals were allocated in three experimental groups that received none (US¯), 1- (1US) or 5-foot-shocks (5US) during fear conditioning and were tested for contextual fear memory. **b** The first batch of animals was tested only twice: 1 and 30 days, while the second batch (**c**) was tested five times: 1, 7, 15, 30, and 50 days after training. **d** A third batch of animals underwent fear conditioning with 5US. **e** Animals were tested 30 days later in two different contexts. Results are present as mean ± SEM. * Indicates difference between groups ($p < 0.05$). # Indicates difference within group ($p < 0.05$)

In addition to the hippocampus, the prefrontal cortex also plays an important role in contextual fear memory, especially regarding remote memory [41–43]. Therefore, we evaluated whether BDNF and Arc mRNA expression, in the PFC, changes 12 h after learning. No alteration in BDNF expression was observed ($F_{(2,\ 15)} = 1.2$; $p = 0.3$; Fig. 3d). Surprisingly, Arc expression was increased in PFC regardless of whether conditioning was done with 1 or 5US ($F_{(2,\ 12)} = 6.2$; $p = 0.01$; Fig. 3e). Altogether, our results indicate that mRNA expression 12 h after training is a molecular marker that differentiates 1US and 5US in the hippocampus, but not in the PFC.

To confirm that hippocampal BDNF and Arc expression 12 h after conditioning depends on de novo RNA synthesis, we took advantage of the RNA synthesis inhibitor actinomycin D (ActD). Mice received ActD in the hippocampus 12 h after learning and 2 h later had their brain removed and processed to analyze mRNA expression of BDNF and Arc (Fig. 3f). 5US increased BDNF expression and, as predicted, ActD prevented such effect (interaction: $F_{(1,\ 16)} = 6.2$; $p = 0.02$; US: $F_{(1,\ 16)} = 4.2$; $p = 0.05$; treatment: $F_{(1,\ 16)} = 5.9$; $p = 0.02$) (Fig. 3g). Similarly, 5US-induced Arc expression was absent after ActD administration (interaction: $F_{(1,\ 14)} = 4.7$; $p = 0.04$; US: $F_{(1,\ 14)} = 4.9$; $p = 0.04$; treatment: $F_{(1,\ 14)} = 5.2$; $p = 0.03$) (Fig. 3h). ActD administered 12 h after 1US conditioning did not change BDNF or Arc expression in the hippocampus, corroborating our results showed in Fig. 2.

To ensure that the dose of ActD used here was inhibiting mainly the learning-induced gene expression, we also measured the expression of the constitutive gene RPL-32. The treatment did not change the RPL-32 expression (interaction: $F_{(1,\ 19)} = 0.3$; $p = 0.5$; US: $F_{(1,\ 19)} = 0.3$; p = 0.5; treatment: $F_{(1,\ 19)} = 2.0$; $p = 0.1$) (Fig. 3i).

Inhibiting Hippocampal De Novo RNA Synthesis 12 h After Training Impaired Remote but not Recent Contextual Fear Memory

To reassure that 12 h is a molecular marker that differentiates recent from remote memory in the hippocampus, we injected ActD directly into the CA1 region of the hippocampus and tested the animals for recent and remote memory (Fig. 4a).

First, we administered intra-hippocampal ActD immediately (0 h) after acquisition and found a recent memory impairment (2 days), in both 1US and 5US groups (interaction: $F_{(1,\ 23)} = 2.6$; $p = 0.1$; US: $F_{(1,23)} = 19.5$; $p = 0.0002$; Treatment: $F_{(1,\ 23)} = 40.2$; $p < 0.0001$) (Fig. 4b). We tested the same animals again, 30 days later. As expected, fear memory was forgotten in animals submitted to 1US. The 5US group that received ActD continued to present less freezing compared to its respective control group which retained the memory (interaction: $F_{(1,\ 22)} = 8.5$; $p = 0.007$; US: $F_{(1,\ 22)} = 18.7$; $p = 0.0003$; treatment: $F_{(1,\ 22)} = 30.1$; $p < 0.0001$) (Fig. 4c).

Next, we administered ActD 12 h after training. No effect of ActD was observed for recent memory, though animals from 5US group froze more than 1US group (interaction: $F_{(1,\ 29)} = 0.09$; $p = 0.7$; US: $F_{(1,\ 29)} = 22.4$; $p < 0.0001$; Treatment: $F_{(1,\ 29)} = 0.09$; $p = 0.7$) (Fig. 4d). As expected, fear memory from 1US mice faded away after 30 days, while 12-h-ActD in 5US group caused remote memory impairment (interaction: $F_{(1,\ 27)} = 24.9$; $p < 0.0001$; US: $F_{(1,\ 27)} = 91.1$;

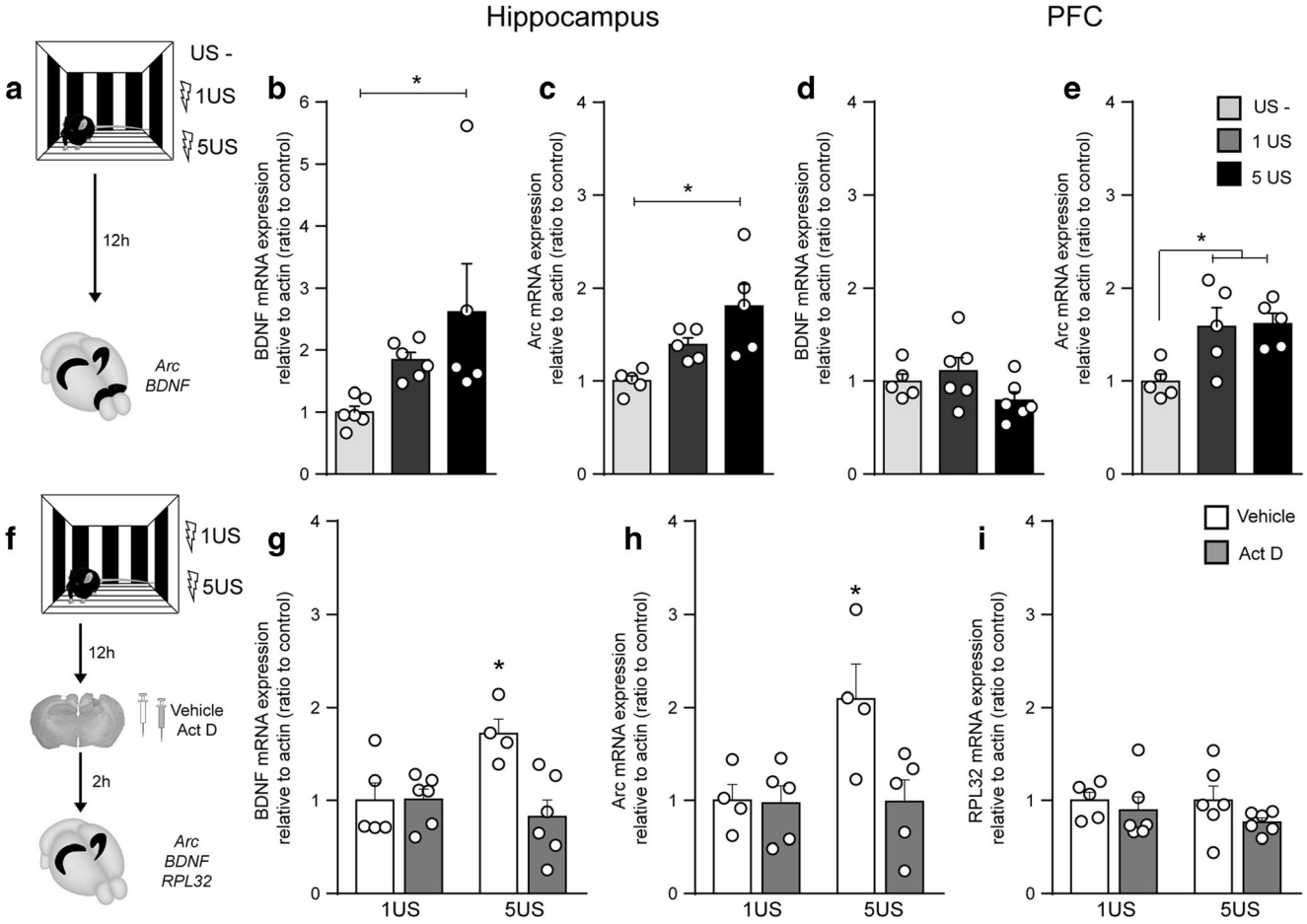

Fig. 3 The mRNA expression of Arc and BDNF is differentially expressed in the hippocampus and prefrontal cortex (PFC) during late consolidation. **a** Animals were allocated in three experimental groups that received none (US^{-}), 1- (1US) or 5-foot-shocks (5US) during fear conditioning, and were euthanized 12 h later. **b** BDNF and **c** Arc expression increased in the hippocampus of animals that underwent to 5US conditioning. No difference was observed in BDNF expression from PFC samples (**d**). **e** Both conditioned groups showed higher expression of Arc in the PFC. **f** A different batch of animals underwent surgery to implant canulae into the CA1 region of the hippocampus. Animals were allocated in three groups: Naïve (control), 1US, and 5US. Twelve hours after conditioning animals received intra-hippocampal injection of either saline or actinomycin (ActD) and 2 h later were euthanized and had their brains processed. Fear conditioning increased (**g**) BDNF and (**h**) Arc expression, while ActD blocked this effect. **i** No difference between groups was observed in the expression of the constitutive gene RPL32. Results are present as mean ± SEM. * Indicates difference between groups ($p < 0.05$)

$p < 0.0001$; treatment: $F_{(1,\ 27)} = 27.1$; $p < 0.0001$ (Fig. 4e). Altogether, our results suggest that 12 h is a molecular marker that differentiates recent from remote memory in the hippocampus.

The Effect of De Novo RNA Synthesis Inhibition, in the dmPFC, Depends on the US Amount

To test whether de novo RNA transcription in the PFC play a functional role on fear memory, we administered ActD directly into the dorsomedial PFC (dmPFC) (Fig. 4a).

Immediately after training, ActD impaired recent memory regardless of whether conditioning was performed with 1 or 5US (interaction: $F_{(1,\ 22)} = 2.2$; $p = 0.1$; US: $F_{(1,\ 22)} = 16.5$; $p = 0.0005$; treatment: $F_{(1,\ 22)} = 16.2$; $p = 0.0006$) (Fig. 4f). When the same 1US animals were tested 30 days after, no remote memory was observed. On contrary, mice treated with ActD immediately after 5US continued to express the memory deficit 30 days later (interaction: $F_{(1,\ 26)} = 11.6$; $p = 0.002$; US: $F_{(1,\ 26)} = 19.6$; $p = 0.0002$; treatment: $F_{(1,\ 26)} = 9.6$; $p = 0.004$) (Fig. 4g).

Unexpectedly, ActD 12 h after training caused opposite effects on recent memory depending on the number of US (interaction: $F_{(1,\ 24)} = 15.7$; $p = 0.0006$; US: $F_{(1,\ 24)} = 84.8$; $p < 0.0001$; treatment: $F_{(1,\ 24)} = 0.1$; $p = 0.7530$). For instance, ActD impaired recent memory caused by 1US, but enhanced the recent memory induced by 5US (Fig. 4h). Again, animals submitted to 1US did not express contextual fear memory 30 days after training. Exacerbated freezing continued to be observed in 5US mice submitted to ActD (interaction: $F_{(1,\ 28)} = 8.8$; $p = 0.006$; US: $F_{(1,\ 28)} = 83.3$; $p < 0.0001$; treatment: $F_{(1,\ 28)} = 4.618$; $p = 0.04$; Fig. 4i).

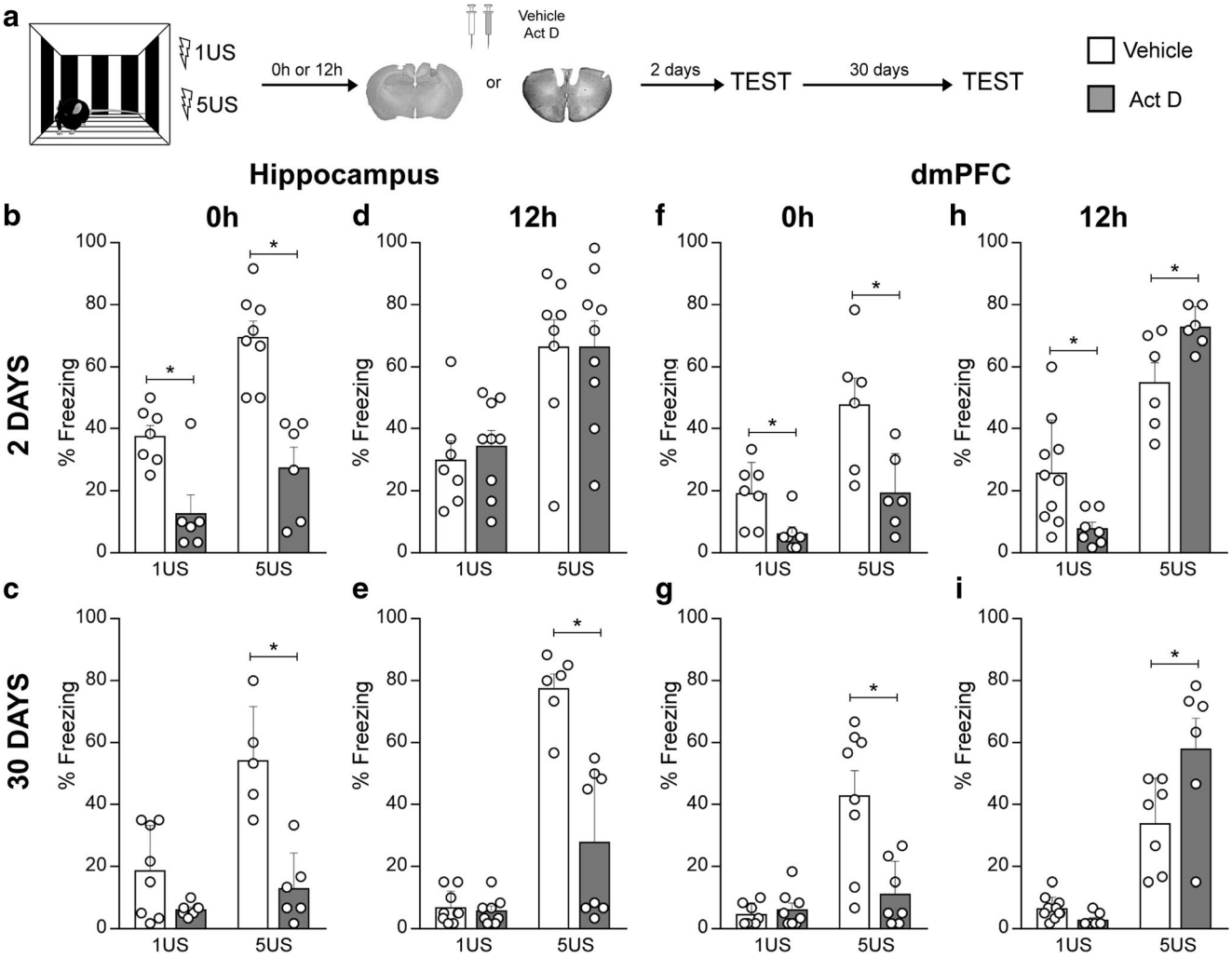

Fig. 4 Inhibition of de novo transcription can either impair or improve contextual fear memory, depending on the number of US and the brain area inhibited. **a** Animals underwent surgery to implant canulae into the CA1 region of the hippocampus or dorsomedial prefrontal cortex (dmPFC). After, mice were conditioned with 1US or 5US and immediately (0 h) or (12 h) after received either saline or actinomycin D (ActD). Contextual fear memory was tested twice: 2 and 30 days after training. Immediately after training intra-hippocampal ActD impaired (**b**) recent and (**c**) remote memories. ActD 12-h post-training does not affect (**d**) recent, but impaired (**e**) remote memory. Immediately after training intra-dmPFC ActD impaired (**f**) recent and (**g**) remote memories. ActD 12 h post has dual effect on (**h**) recent memory and (**i**) exacerbated remote memory. Results are present as mean ± SEM. *Indicates difference between groups ($p < 0.05$)

Our results indicate that 0 h after training is a critical time point to fear memory formation in the dmPFC as it is for the hippocampus. However, unlike the hippocampus, 12 h is a critical time point for recent memory consolidation in the dmPFC. Interestingly, inhibition of de novo RNA synthesis at 12 h post-training leads to opposite effects on fear behavior, depending on the number of foot-shocks delivered during training.

Exacerbated Fear Behavior Triggered by the De Novo RNA Inhibition into the dmPFC Can Be Extinguished and Did Not Generalize

We observed that if administered into the dmPFC, 12 h after acquisition, ActD increases freezing behavior in animals tested 2 days (recent memory) and 30 days (remote memory) later. Interestingly, higher freezing was observed only in animals that underwent conditioning with 5US. To characterize the exacerbated fear behavior that occurred after combining intra-dmPFC ActD and 5US conditioning, we subjected a different group of animals to an extinction protocol (Fig. 5a). Recent memory was extinguished in both vehicle and ActD groups (interaction: $F_{(3, 42)} = 4.3$; $p = 0.009$; session: $F_{(3, 42)} = 56.9$; $p < 0.0001$; treatment: $F_{(1, 14)} = 16.6$; $p = 0.001$) (Fig. 5b). For remote memory, the same pattern was observed (interaction: $F_{(3, 45)} = 6$; $p = 0.001$; session: $F_{(3, 45)} = 187.2$; $p < 0.0001$; treatment: $F_{(1, 15)} = 6$; $p = 0.02$) (Fig. 5d).

Despite our results indicate the exacerbated fear memory is extinguishable with the same number of re-exposure sessions, ActD groups showed higher initial levels of fear. Therefore, we

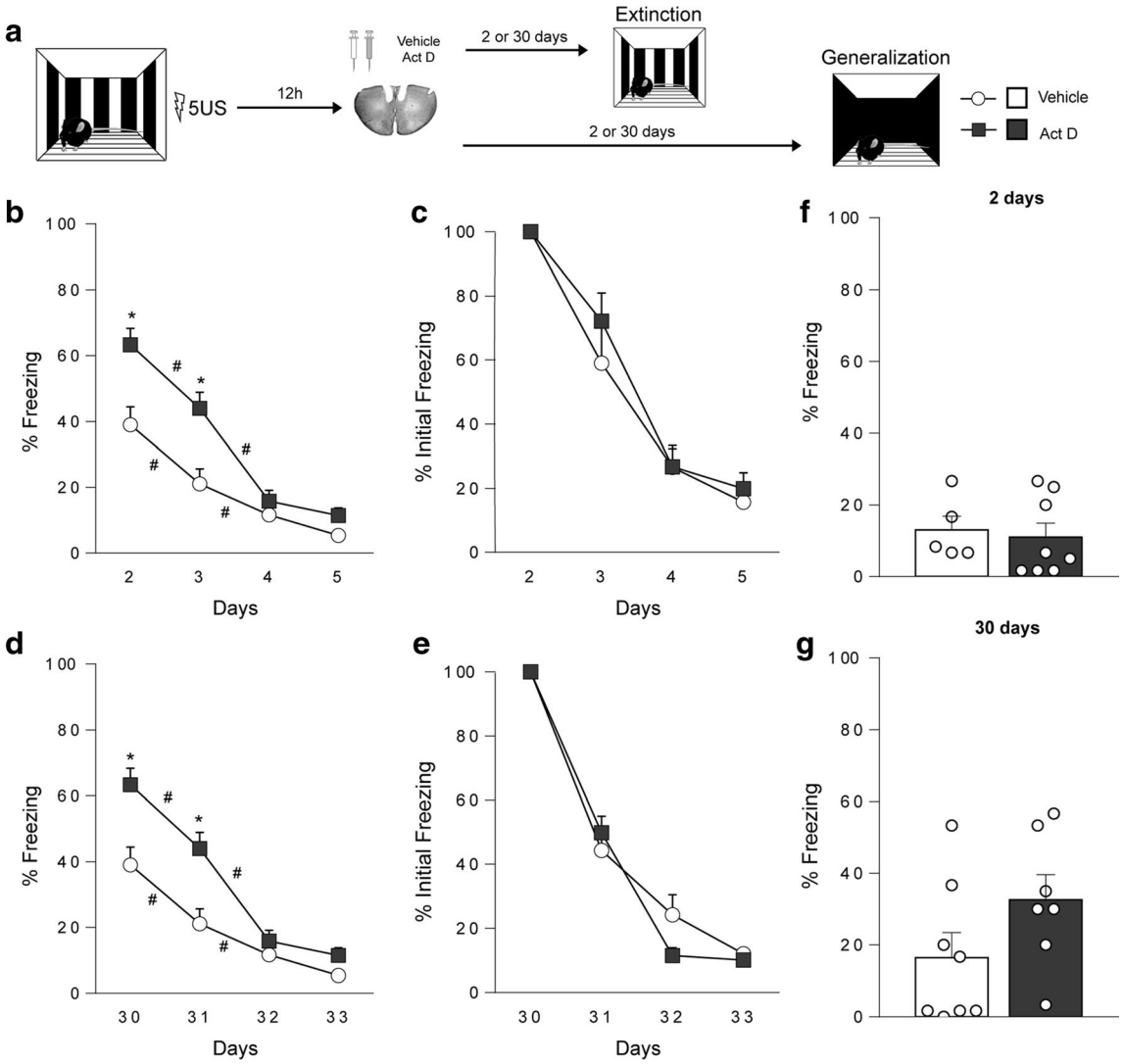

Fig. 5 Contextual fear memory triggered by inhibiting de novo transcription into the dmPFC, 12 h after 5US conditioning, is susceptible to extinction and does not generalize. **a** Animals underwent surgery to implant canulae into the dorsomedial prefrontal cortex (dmPFC). After, mice were conditioned with 5US and (12 h) after received either saline or actinomycin D (ActD). **b**, **c** One batch of animals was tested 2 days after training and for another 3 days (3, 4, and 5 days). **d**, **e** A second batch of animals was tested 30 days after training (30 days) and for another 3 days (31, 32, and 33 days). In the generalization experiment, a third batch of mice was tested in a different context either **f** 2 or **g** 30 days after training. Results are present as mean ± SEM. *Indicates difference between groups ($p < 0.05$). # Indicates difference within group ($p < 0.05$)

examined the relative extinction rates, by calculating the rate of extinction by percent of initial freezing [44]. Relative extinction rate did not differ between groups, for both recent (Fig. 5c: interaction: $F(3,42) = 0.5$; $p = 0.6$; time: $F(3,42) = 77.5$, $p < 0.0001$; treatment: $F(1,14) = 0.06$, $p = 0.4$) and remote (Fig. 5e: interaction: $F(3,45) = 1.7$, $p = 0.2$; time: $F(3,45) = 1.9$, $p < 0.0001$; treatment: $F(1,15) = 0.7$, $p = 0.4$) memory.

Next, we tested whether these exacerbated fear responses were a product of overgeneralization. Animals were submitted to 5US conditioning and treated with ActD or vehicle in dmPFC 12 h after learning and tested in a different context, 2 or 30 days after conditioning (Fig. 5a). Here, we choose a protocol that usually do not cause fear generalization [23], to verify whether our condition would be inducing a traumatic fear memory. Animals did not generalize 2 ($t_{(11)} = 0.3$; $p = 0.7$) (Fig. 5f) or 30 days after learning ($t_{(13)} = 1.6$; $p = 0.1$) (Fig. 5g). Thus, we may conclude that the increased freezing behavior was not associated with extinction impairment or overgeneralization, in conditions where it is expected that animals do not generalize.

Inhibition of De Novo RNA Synthesis in dmPFC 12 h After Fear Learning Intensifies Amygdala Activation

Unlike the hippocampus, inhibiting de novo RNA synthesis in the dmPFC during late consolidation affected both recent and

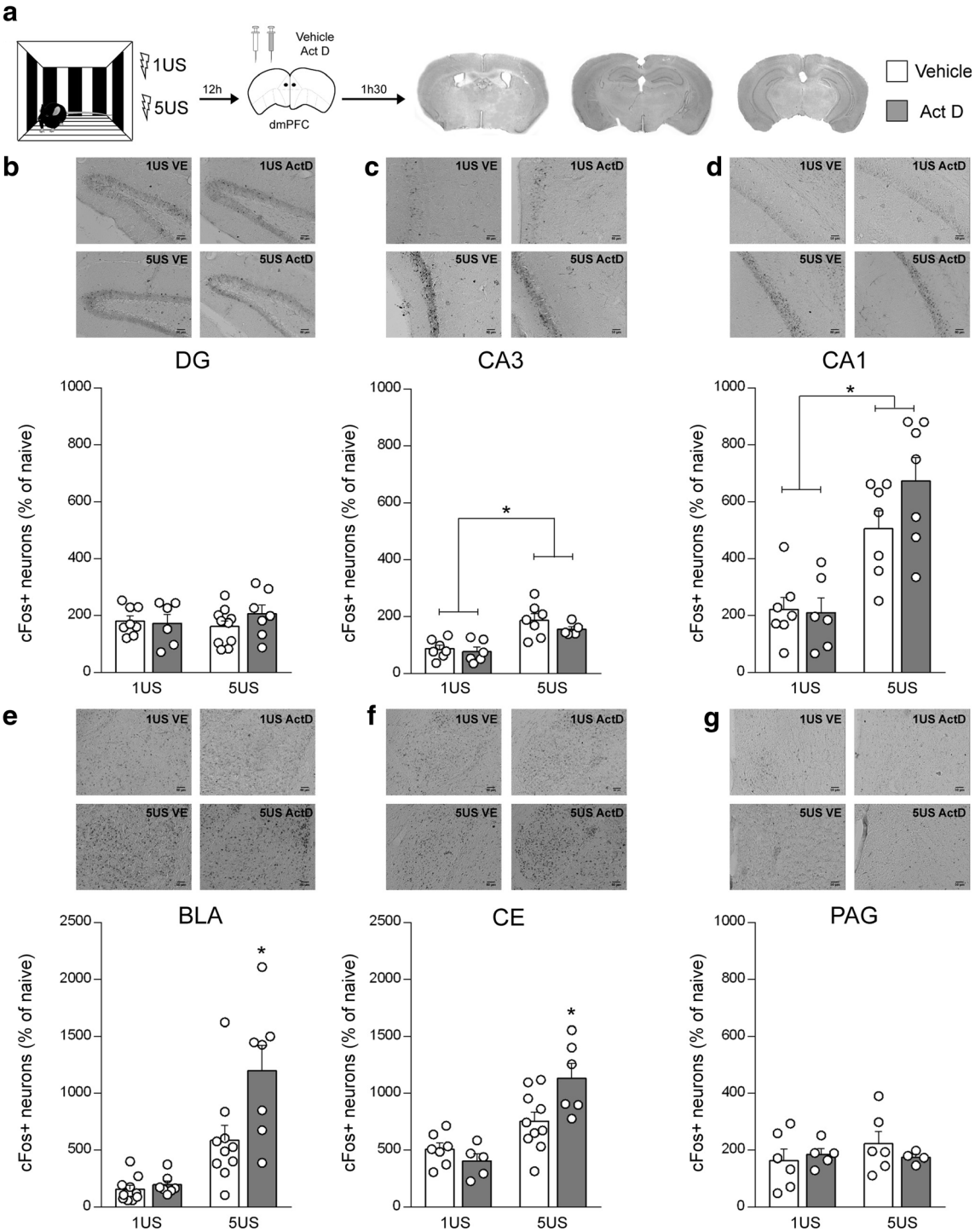

Fig. 6 Inhibition of de novo transcription into the dmPFC 12 h after training specifically increased BLA and CE activity in animals conditioned with 5US. **a** Animals were allocated in three groups: Naïve (control), 1US, and 5US. Animals from 1US and 5US underwent surgery to implant canulae into the dorsomedial prefrontal cortex (dmPFC). Twelve hours after conditioning, animals received intra-dmPFC injection of either saline or actinomycin (ActD) and 1 h 30 min later were euthanized and had their brains processed for immunohistochemistry. At the same time, naïve animals were removed from their home cages and were euthanized. Representative images and $cFos^{+}$ neurons (% of naïve) quantification in the **b** dentate gyrus (DG), **c** CA3, **d** CA1 regions of the hippocampus, **e** basolateral amygdala (BLA), **f** central amygdala (CE), and **g** periaqueductal gray (PAG). Results are present as mean ± SEM.* Indicates difference between groups ($p < 0.05$)

remote memories. Accordingly, Arc expression was increased 12 h after conditioning with either 1US or 5US. Although it is not a critical time point for the differentiation of memories, gene transcription in dmPFC 12 h after fear conditioning

seems to be decisive for the memory's fate: being forgotten or remembered. Considering the top-down modulation exerted by dmPFC over fear-related brain areas [45, 46], we decided to address whether ActD administration into the dmPFC 12 h would be altering the activity of brain areas important to fear memory processing.

Animals were submitted to CFC (1US or 5US) and 12 h later received either vehicle or ActD into the dmPFC. Then, 1 h 30 min after, animals were euthanized and their brains used for c-Fos immunohistochemistry (Fig. 6a).

In the dentate gyrus of the hippocampus (DG), we did not observe differences between groups (interaction: $F_{(1, 27)} = 1.1$; $p = 0.3$; US: $F_{(1, 27)} = 0.5$; $p = 0.4$; treatment: $F_{(1, 27)} = 0.06$; $p = 0.7$) (Fig. 6b). CA3 region of 5US animals showed higher number of c-Fos positive neurons, though ActD had no effect (interaction: $F_{(1, 21)} = 0.4$; $p = 0.5$; US: $F_{(1, 21)} = 27.9$; $p < 0.0001$; treatment: $F_{(1, 21)} = 1.5$; $p = 0.2$) (Fig. 6d). In Fig. 6e, we can observe that 5US also increased c-Fos expression in the CA1 region, although no effect of treatment was found (interaction: $F_{(1, 22)} = 0.01$; $p = 0.9$; US: $F_{(1, 22)} = 23.2$; $p < 0.0001$; treatment: $F_{(1, 22)} = 0.08$; $p = 0.7$).

Next, we analyzed the pattern of activation in the amygdala. The inhibition of de novo transcription in the dmPFC following the 5US training increases the number of c-Fos positive cells in the basolateral (BLA) (interaction: $F_{(1, 24)} = 3.3$; $p = 0.08$; US: $F_{(1, 24)} = 21.7$; $p < 0.0001$; treatment: $F_{(1, 24)} = 4.3$; $p = 0.04$) (Fig. 6e) and central (CE) (interaction: $F_{(1, 24)} = 7.1$; $p = 0.01$; US: $F_{(1, 24)} = 29.1$; $p < 0.0001$; treatment: $F_{(1, 24)} = 2.2$; $p = 0.1$) (Fig. 6f) region of amygdala.

Finally, in periaqueductal gray (PAG), we did not observe any difference between groups (interaction: $F_{(1, 17)} = 0.9$; $p = 0.3$; US: $F_{(1, 17)} = 0.4$; $p = 0.5$; treatment: $F_{(1, 17)} = 0.1$; $p = 0.7$) (Fig. 6g).

Our results suggest that 5US induce neuronal activation at 12 h post-training, in areas that process fear. We may also suggest that the amygdala's activation is particularly sensitive to the inhibition of transcription in the dmPFC 12 h after 5US-conditioning.

Discussion

We first sought to establish a protocol to dissociate recent and remote memories and found that by altering the number of foot-shocks (US), fear memory can be limited to 24 h or last for 30 days in C57/BL6 mice. Others have shown that fear memory recall can be extended by exposing mice [17] and rats [23, 47] to training sessions with more foot-shocks, or by intensifying the shock's amplitude [48, 49]. Specifically, here, we showed that 5US induces remote contextual fear memory. Furthermore, fear memory decayed across extinction trials and animals did not generalize, suggesting that the memory trace formed by 5US is context-specific and susceptible to extinction.

As showed in rats [23], we found that mice froze more after being conditioned with 5US compared to 1US, and additionally, we showed the increased freezing behavior is sustained even after 30 days. Furthermore, mice did not generalize 30 days after 5US conditioning. Some studies showed that fear generalization may occur 28 days after contextual conditioning [40, 50, 51], though 1US was used and not a stronger training protocol, such as the one used here.

We predicted that 5US may induce an additional molecular change that allows memory to persist for 30 days. We focused on 12 h post-training because previous studies had indicated as a critical time point to consolidation of remote memory in the hippocampus [15, 16, 33]. As expected, we showed that BDNF and Arc are upregulated in the hippocampus, 12 h after 5US, but not 1US. By using a distinct paradigm, protocol and species, our results add evidence to support that BDNF and Arc expression are molecular markers of remote memory.

The transcription of genes related to synaptic plasticity, such as BDNF and Arc, was triggered by 5US at a late consolidation time point. Thus, we asked whether this result would have any relation to contextual fear memory persistence. We revealed that 12-h inhibition of gene transcription in CA1 region of the dorsal hippocampus (dHIP) impaired remote, but not recent memory, which is similar to what occurs in rats [16]. Additionally, we showed that immediately after training, gene transcription in dHIP is necessary for the formation of recent and remote memories, as supported by previous work [17, 27, 52]. Altogether, we observed a cause-effect relationship between 12-h post training mRNA expression and remote memory in the hippocampus, indicating that this is a critical time point to molecularly differentiate recent and remote contextual fear memory in the dHIP of mice.

Motivated by data showing prefrontal cortex (PFC) recruitment during fear memory consolidation and retrieval [53, 54], we evaluated whether gene transcription in PFC would be changed by 1US or 5US 12 h after training. In a recent study, authors identified a range of mRNAs that are upregulated in the PFC, 1 h after contextual fear conditioning with 3US, and showed that mRNA transcription is required for encoding [55], but no analysis was performed 12 h after. Opposite to what occurs in the hippocampus, no changes in BDNF expression were observed in PFC. We also found that Arc expression increased after both 1US and 5US, suggesting that 12-h RNA transcription is not a molecular marker to differentiate recent from remote memory in the PFC. Nevertheless, we may not rule out that 12 h is a critical time point to late consolidation in the PFC. In fact, medial prefrontal cortex activation of D1/D5 dopamine receptors, 12 h post-training, is essential for remote, but not recent inhibitory avoidance memory in rats [49].

To further investigate whether gene transcription in PFC would play a role in contextual fear memory, we inhibited de novo RNA transcription into the dmPFC. ActD administered intra-dmPFC, immediately after training impaired recent and remote memory just as it did in the dHIP. These results suggest that gene transcription in the dmPFC, during early consolidation is important for recent and remote memories, which is in accordance with previous work [55, 56].

We showed for the first time that, contrary to the dHIP, inhibiting gene transcription 12 h post-training in the dmPFC profoundly affected recent memory. While 1US group have their memory impaired by ActD, fear behavior increased in 5US group. The dual effect of ActD on fear memory is not explained by Arc expression in the dmPFC, because 1US and 5US groups exhibited similar expression levels. Therefore, any attempt to explain this result will fall within the scope of speculation. However, the present results are shining light to the variables strength of training and late consolidation time, which are usually overlooked in the field of prefrontal cortex role on remote memory.

In attempt to better understand the dual effect of intra-dmPFC ActD administration, we quantified c-Fos expression as a proxy for neural activity [8] in the brain areas of the fear circuit [57, 58] 12 h post-conditioning. We predicted that disturbing molecular events dependent on de novo transcription in the dmPFC would affect the activity of brain areas modulated by such events, compromising the expression of conditioned fear during retrieval. We found increased c-Fos expression in both BLA and CE, specifically after the combination of 5US and intra-dmPFC ActD. Interestingly, mice exposed to this condition were the ones with enhanced recent and remote fear memory. These results suggest that dmPFC-amygdala circuits may play a role on shaping contextual fear memories induced by strong training, such as 5US.

In dmPFC, 4-Hz oscillations are generated by parvalbumin positive inhibitory interneurons (PV neurons) [59] and drive BLA activity during freezing expression [60]. In fact, it seems that there is a sustained oscillatory mechanism mediating prefrontal-amygdala coupling during fear behavior [60]. One conceivable mechanism to explain our results could involve the dmPFC-BLA oscillations. In other words, dmPFC-BLA coupling may occur 12 h after acquisition of contextual fear and ActD would be disturbing this process. Similar idea was observed in ventral hippocampus. At 12 h after contextual fear conditioning with 5US, PV plasticity was required for enhanced sharp-wave ripple densities and c-Fos expression in pyramidal neurons [32].

No specific effect of ActD on c-Fos expression was observed after 1US. However, animals from 1US group had their recent memory impaired after ActD administration. Interestingly, optogenetic inhibition of dmPFC parvalbumin-expressing interneurons in the ascending phase of 4-Hz oscillations reduced freezing, while the same manipulation, though in the descending phase, increased freezing [61]. Thus, we may speculate that the dual effect of intra-dmPFC ActD could be an outcome of the drug's effect on dmPFC 4-Hz oscillation.

Furthermore, there are bidirectional projections between BLA and mPFC. BLA neurons specifically synapse with mPFC neurons that project to PAG [62]. Therefore, by injecting ActD into the dmPFC, we can virtually be affecting BLA projections, for example, in addition to dmPFC neurons.

Recent evidence suggests that anterior and posterior parts of the PFC may produce distinct behavioral effects in an active-avoidance paradigm [63]. In the present study, we do not have sufficient data to separate the behavioral analysis based on cannula positioning in the rostral-caudal plane. However, it would be very interesting in the future to design experiments to approach specifically this issue, in contextual fear memory.

PAG did not display differences in c-Fos expression, as predicted, since this area is more related to fear expression than memory consolidation [64, 65]. However, in the hippocampus, 5US increased c-Fos expression in CA1 and CA3 regions, regardless of the treatment. The fact that the hippocampus is still active 12 h post-5US is in accordance with others [32, 66]. One of the possible explanations for the c-Fos late expression is that the memory engram is being recruited once again, leading to replay processes that occur during wakefulness and non-REM sleep [67–70]. As we also observed a c-Fos late expression triggered by 5US, in the amygdala, our study suggests that replay events may also occur in areas other than the hippocampus.

Collectively, our data highlights the importance of late molecular events to contextual fear memory formation and persistence. Our study also rises important questions regarding the bidirectional effect of dmPFC transcripts on fear memory, at a specific time point of late consolidation.

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