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Social experiences switch states of memory engrams through regulating hippocampal Rac1 activity

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In pathological or artificial conditions, memory can be formed as silenced engrams that are unavailable for retrieval by presenting conditioned stimuli but can be artificially switched into the latent state so that natural recall is allowed. However, it remains unclear whether such different states of engrams bear any physiological significance and can be switched through physiological mechanisms. Here, we show that an acute social reward experience switches the silent memory engram into the latent state. Conversely, an acute social stress causes transient forgetting via turning a latent memory engram into a silent state. Such emotion-driven bidirectional switching between latent and silent states of engrams is mediated through regulation of Rac1 activity–dependent reversible forgetting in the hippocampus, as stress-activated Rac1 suppresses retrieval, while reward recovers silenced memory under amnesia by inhibiting Rac1. Thus, data presented reveal hippocampal Rac1 activity as the basis for emotion-mediated switching between latent and silent engrams to achieve emotion-driven behavioral flexibility.

engram | Rac1 | social reward | social stress | forgetting

Significance

It is well known that silent memory engrams in pathological or artificial conditions can be artificially switched into the latent state for retrieval by natural recall cues. Thus, physiological strategies that depend on the underlying molecular mechanisms for switching between silent state and latent state are a subject for investigation. Here, we show that social experiences stimulated switching between latent and silent engrams to achieve flexible memory accessibility and also reveal the basic molecular mechanism of: 1) social reward turning silent engram to latent via suppression of Rac1 activity in CA1 neurons of the hippocampus; and 2) social stress switching latent memory engram into silent through activating Rac1. Together, this work demonstrates emotion-driven bidirectional switching between latent and silent engrams.

Animals are required to flexibly retrieve memories according to their emotional states for achieving optimal behaviors in an ever-changing environment. To understand the underlying mechanisms of such regulation, extensive effort has been devoted to studying the impact of emotion on memory processes (1–5). For instance, stress can block memory retrieval through hormones, neuroinflammation, or depression of synapses in both human and animal models (6–12), while reward and novelty can facilitate both the formation and maintenance of memories (13, 14). However, how emotion might directly impact the memory engram remains elusive. The proposed theory and experimental demonstrations have revealed the presence of multiple states of memory engrams, such as silent, latent, and active states (15–17). In the silent state, only artificial activation of engram cells is capable of inducing memory expression, whereas latent engram cells can be activated by a natural conditioned stimulus to drive the engram into the active state for memory retrieval. It is intriguing to note that training either mouse models of Alzheimer's disease or protein synthesis-inhibited mice are reported to yield only silent engrams, and such silent engrams could be turned into a latent state (18–22) through artificial manipulations of either optical stimulation–induced long-term potentiation (LTP) or virus-driven overexpression of activated PAK1. However, the physiological significance of the different states of engrams, particularly the silent engram, remains unclear. In the course of studying the functions of reversible forgetting (23, 24), we became interested in testing the idea that the emotional impact on memory retrieval could be mediated through switching the engram between latent and silent states, while reversible forgetting may play a role in making such switching.

To investigate this idea, we tested the effects of acute social reward (SR) and social stress (SS) on memory retrieval. Since mating and fighting are widely perceived and used as behavioral stimuli for evoking feelings of reward and stress, respectively (25–27), we adopted a modified short procedure for evoking acute emotion through social interactions. For SR treatment, a single experimental male mouse is exposed to two females brought from different home cages for 10 min. Such a subtle positive experience is sufficient to enhance retrieval of contextual fear memory (28). For SS treatment, a single experimental male mouse is exposed to a group of five male littermates for 10 min. This is a hostile social environment in which the experimental mouse fights with other littermates during this time window. Such a subtle stressful experience is sufficient to significantly reduce 24-h contextual fear memory (28). Based on these two paradigms of acute social experiences, we investigated how emotion affects memory retrieval through alterations in engram states.

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Results

Social Reward Could Temporarily Switch the Silent Engram into the Latent State. We first tested whether SR could switch protein synthesis inhibition–silenced engrams into the latent state so that the amnesic memory could be retrieved through natural cues. It is reported that injection of the protein synthesis inhibitor anisomycin (ANI) immediately after contextual fear conditioning (CFC) leads to retrograde amnesic memory (29) by silencing engram cells, which can be artificially activated through optogenetic stimulation to retrieve amnesic memory (19, 21). As expected, five-trial CFC-induced 24-h contextual fear memory was disrupted by posttraining administration of ANI in controls (Fig. 1 *A* and *B*). However, when the conditioned mouse was presented with SR treatment immediately before the 24-h memory test (Fig. 1*A*), the amnesic contextual fear memory could be retrieved by natural recall cues, which was also observed in cycloheximide (CXM, another protein synthesis inhibitor)-induced amnesic memory (*SI Appendix*, Fig. S1 *A* and *B*), and such SR-mediated recovery of the amnesic memory was temporary in nature, as this recovery diminished 24 h after the first recall (Fig. 1*B*). In addition, the presentation of SR was ineffective when given 2 h before memory recall (*SI Appendix*, Fig. S2 *A* and *B*).

To gain insights into the effects of SR on the engram state that allowed the recovery of the amnesic memory, we examined engram reactivation in the CA1 (Cornu Amonis) region of the hippocampus with regard to the retrieval of the 24-h memory (Fig. 1*C*) because retrievability by natural recall cues relies on the states of these engrams (17, 18, 20, 21). We injected the adeno-associated viruses $(AAV)_{2/9}$-c-fos:tetracycline-controlled transactivator (tTA) and $AAV_{2/9}$-tetracycline response element (TRE)-tandem dimer tomato (tdTomato) (Fig. 1*D*) into the CA1 region to label learning-induced engram cells (19, 24), and then detected the recall-stimulated c-Fos$^+$ cells by c-Fos staining (Fig. 1*D* and *SI Appendix*, Fig. S3*A*). We found that the number of activated latent engram cells resulting from natural recall was significantly reduced in ANI-injected mice (ANI in Fig. 1*E*) as compared to the saline-injected control mice (SAL in Fig. 1*E*). To describe this observation more rigorously, the ratio of reactivated engram cells (c-fos$^+$ tdTomato$^+$ double positive) to learning-activated total engram cells (tdTomato$^+$ staining) was decreased by ANI injection (Fig. 1*F*). SR treatment recovered the number of latent engram cells, which are available for natural recall (ANI+SR in Fig. 1*E*) and the reactivation ratio of engram cells in CA1 of the ANI-injected mice (Fig. 1*F*), while SR without recall has no effect on the reactivation of engram cells (*SI Appendix*, Fig. S4). In nonengram cells (tdTomato$^-$ cells: DAPI without tdTomato signal), SR had no impact on the ratio of c-fos$^+$ tdTomato$^-$ cells to tdTomato$^-$ cells (Fig. 1*F*). Consisitent with behavioral data, the effect of SR in the reactivation of the engram cells in CA1 was also temporary and diminished 24 h after the first recall (Fig. 1*G* and *SI Appendix*, Fig. S5*A*). To confirm this observation, we also examined the effect of SR on contextual fear memory induced by single-shock CFC and found that SR showed a similar rescue effect on ANI-induced amnesic memory

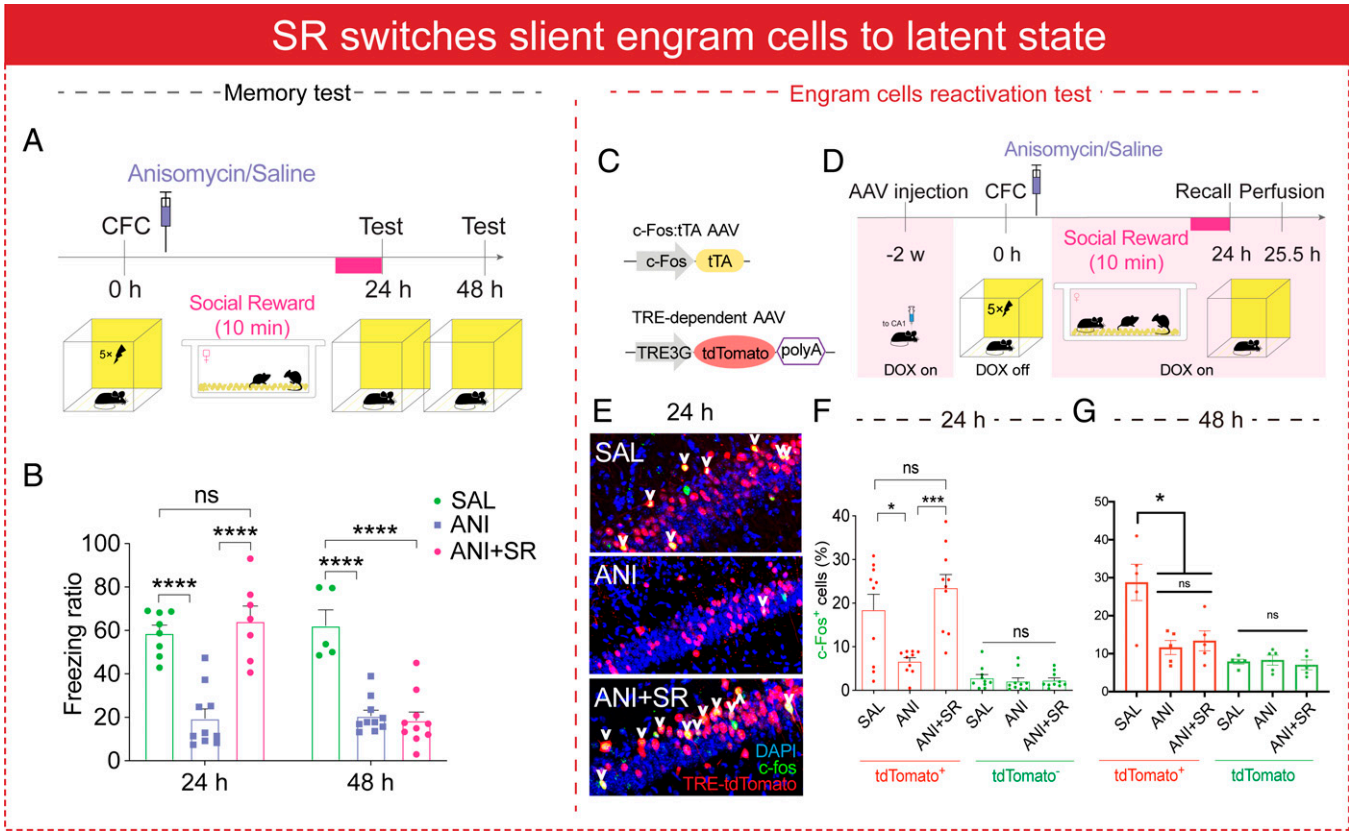

Fig. 1. Acute social reward switches silent memory engram into a latent state. (*A*) Experimental schedule. (*B*) The effects of SR on contextual fear memory under protein synthesis inhibitor-induced amnesia (24 h: $n_{SAL} = 8$, $n_{ANI} = 10$, $n_{ANI+SR} = 7$ 48 h: $n_{SAL} = 5$, $n_{ANI} = 10$, $n_{ANI+SR} = 10$). (*C* and *D*) Strategy of hippocampus engram overlap and CFC. (*E*) Coronal section of CA1 engram cells (TRE-tdTomato) labeling with anti–c-fos (green). (Scale bar, 50 μm.) Arrowheads indicate double positive. (*F and H*) Percentages of c-Fos$^+$ cells in tdTomato$^+$ and tdTomato$^-$ cells in CA1. (*F*) 10 slices from five mice for each group and (*H*) 5 slices from five mice for each group. ns $P > 0.05$, *$P < 0.05$, ***$P < 0.001$, and ****$P < 0.0001$; from one-way ANOVA with Turkey test (*F and G*) and two-way ANOVA with Bonferroni test (*B*). Data are presented as means ± SEM.

(*SI Appendix*, Fig. S6). Combining the behavioral observations with the detection of reactivated engram cells, our data support the notion that SR treatment switches the protein-synthesis inhibition-silenced engram into the latent state.

Social Stress Could Temporarily Switch a Latent Engram into a Silent State. Given that acute stress was sufficient to reduce memory expression in both animal models and human (6, 7, 10), we next tested whether SS could inhibit memory retrieval by switching latent engrams to the silent state. For this purpose, a male mouse was subjected to five-trial CFC. Immediately before the 24-h memory test, the conditioned mouse was exposed to a group of five hostile male littermates (Fig. 2*A*). We found that 24-h memory retrieval was indeed temporarily disrupted by SS treatment, as a spontaneous recovery of memory retrieval was observed 24 h later (Fig. 2*B*). Consistent with the effect of SR, the presentation of SS was also ineffective when given 2 h before memory recall (*SI Appendix*, Fig. S2 *C* and *D*). Subsequently, we examined the effect of SS on the reactivation of engram cells during retrieval (Fig. 2*C*). Consistently, the ratio of reactivated engram cells (c-fos$^+$ tdTomato$^+$ double positive) (*SI Appendix*, Fig. S3*B*) to learning-activated total engram cells (tdTomato$^+$ staining) was decreased by SS treatment (Fig. 2 *D* and *E*), while SS without recall has no effect on the reactivation of engram cells (*SI Appendix*, Fig. S4). In nonengram cells, there was no difference in the ratio of c-fos$^+$ tdTomato$^-$/tdTomato$^-$ for each group (Fig. 2*E*). Consistent with behavioral data, the effect of SS in the reactivation of the engram cells in CA1 was also temporary and diminished 24 h after the first recall (Fig. 2*E* and *SI Appendix*, Fig. S5*B*). Taken together, these data suggest a crucial role of acute social–emotional experiences in regulating states of the memory engram, as SR reverses the protein synthesis inhibition-silenced memory engram back into the latent state, while SS does not erase memory.

Hippocampal Rac1 Activity Correlates with Latent and Silent Memory Engram States. In pursuing molecular mechanisms underlying such switching between latent to silent states, we were drawn to the idea of involvement of Rac1-dependent reversible forgetting, as elevated Rac1 activity in the hippocampus leads to the forgetting of memories in multiple tasks (23, 24, 30–32), and the loss of the memory is reversed when Rac1 activity is suppressed (24). Given that multiple-trial CFC led to suppression of Rac1 activity (24), it is possible that protein synthesis inhibition disrupts the suppression of Rac1 activity induced by multiple-trial CFC so that the increase of Rac1 activity subsequently leads to reversible amnesia. To test this idea, ANI was injected (intraperitoneally [i.p.]) immediately after CFC (Fig. 3*A*). By immunoblotting active GTP-bound Rac1 (Rac1-GTP) in the hippocampus, we found that Rac1 activity was inhibited by five-trial CFC in controls (injected with saline), assayed 24 h after conditioning, but dramatically elevated in ANI-injected mice (Fig. 3 *B*–*E*). To confirm the causal link between elevated Rac1 activity and amnesia, we manipulated the Rac1 activity in the dorsal CA1 through injection of AAVs, which carries a mutant transgene that encodes the dominant-negative Rac1 (Rac1-DN) targeted to excitatory neurons via the CaMKIIα promotor (*SI Appendix*, Fig. S7*A*). Such expression has been shown to inhibit evoked Rac1 activity (23, 24, 31). Two weeks after the surgery, the mice were

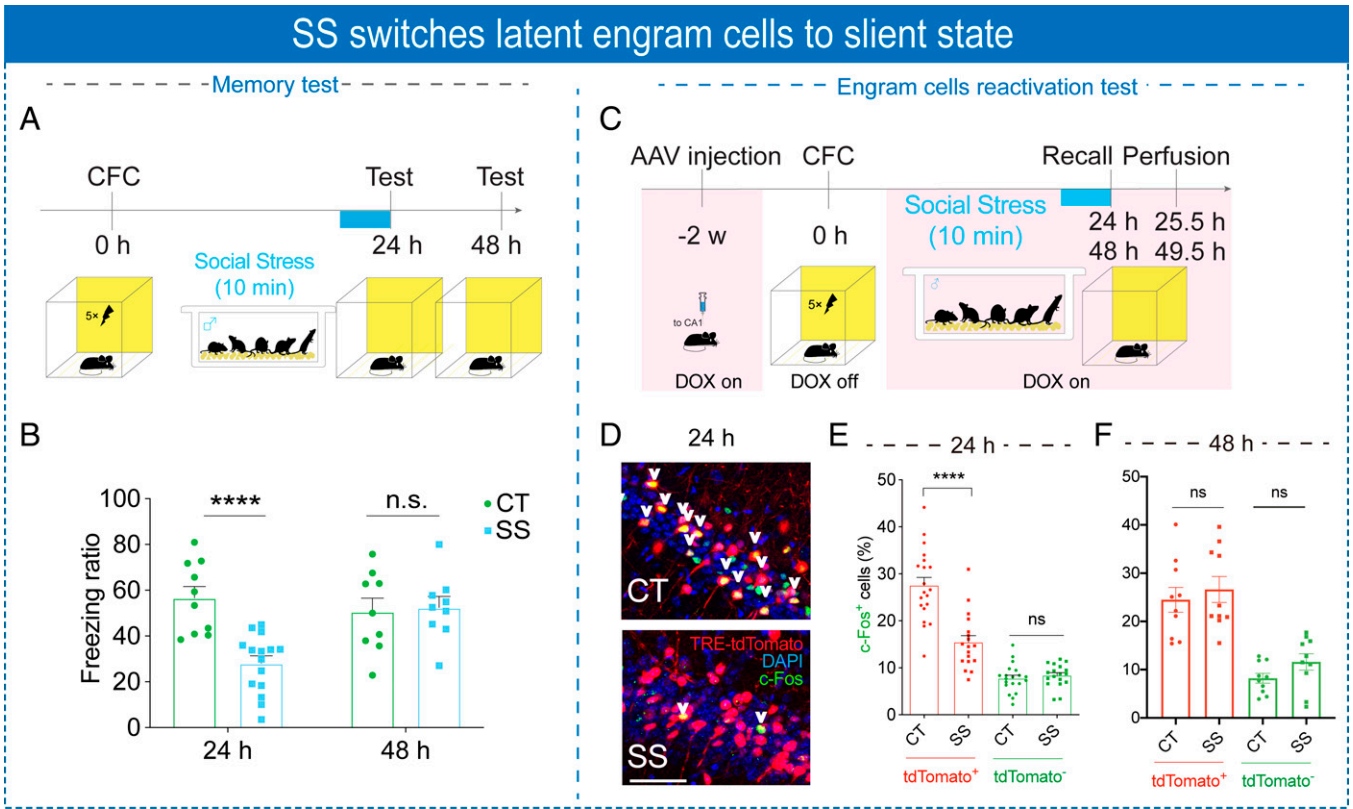

Fig. 2. Acute social stress converts latent engrams to silent state. (*A*) Experimental schedule. (*B*) The effects of SS on contextual fear memory (24 h: n_{CT} = 10, n_{SS} = 15 48 h: n_{CT} = 9, n_{SS} = 9). (*C*) Strategy of hippocampus engram overlap and CFC. (*D*) Coronal section of CA1 engram cells (TRE-tdTomato) labeling with anti–c-fos (green). (Scale bar, 50 μm.) Arrowheads indicate double positive. (*E* and *F*) Percentages of c-Fos$^+$ cells in tdTomato$^+$ and tdTomato$^-$ cells in CA1 at 24 h (*E*) or 48 h (*F*) after training. (*E*) 18 to 20 slices from five mice for each group. ns $P > 0.05$, ****$P < 0.0001$; from unpaired *t* test (*E* and *F*) and two-way ANOVA with Bonferroni test (*B*). Data are presented as means ± SEM.

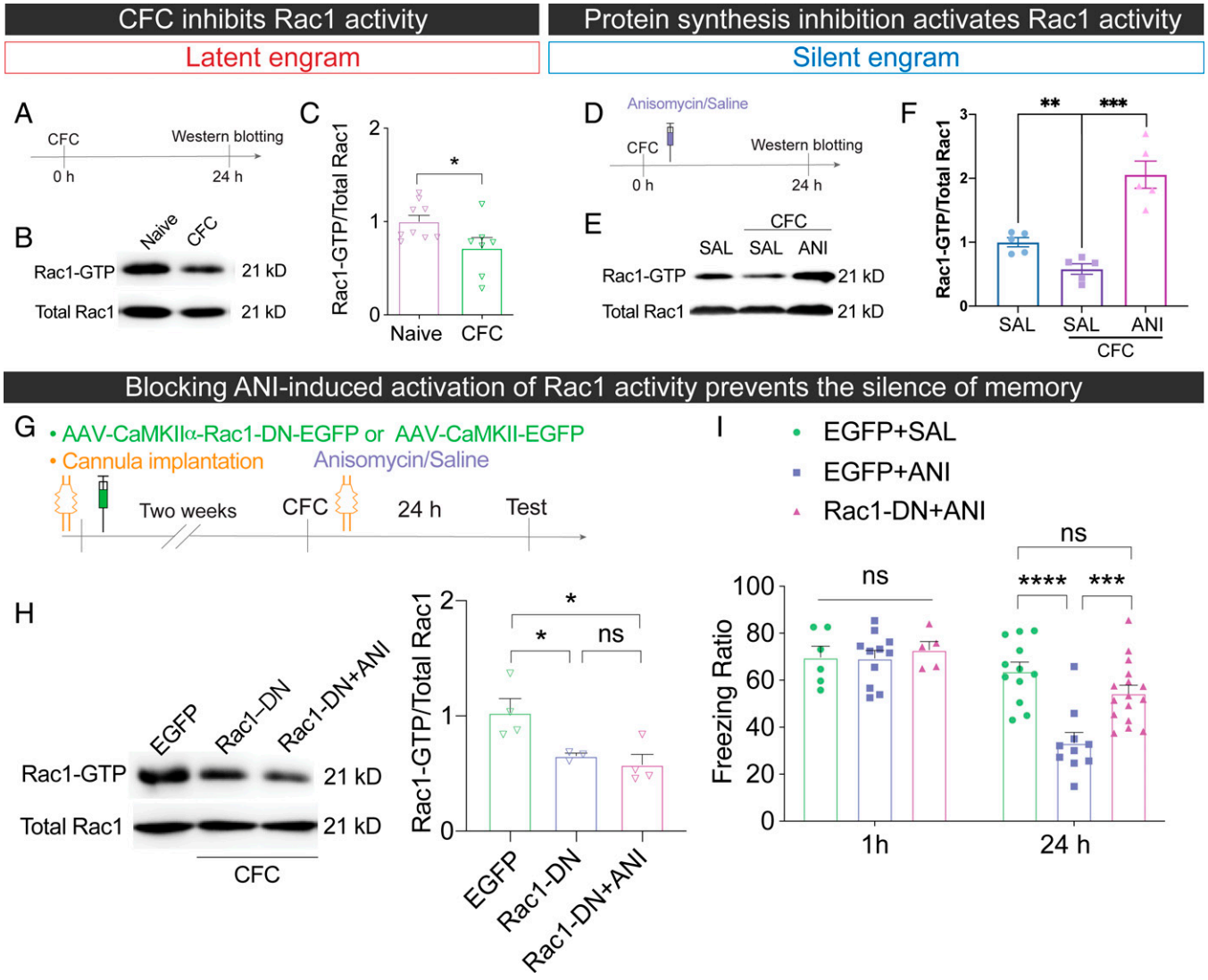

Fig. 3. Hippocampal Rac1 activity correlates with latent and silent memory engram states. (*A*, *D*, *and G*) Experimental schedule. (*B*, *C*, *E*, *F*, and *H)* Immunoblotting and data showing Rac1 activity (Rac1-GTP) and total Rac1 levels in hippocampus [normalization of the value of Rac1-GFP/total Rac1: (*C*) Normalized to the naive group; (*F*) normalized to the SAL group; (*H*) normalized to the EGFP group] at 1 d after CFC. (*C*) $n_{Naive} = 11$, $n_{CFC} = 7$; (*F*) $n_{Naive} = 5$, $n_{SAL} = 5$, $n_{ANI} = 5$; and (*H*) $n_{Ctrl} = 4$, $n_{Rac1\text{-}DN} = 3$, $n_{Rac1\text{-}DN+ANI} = 4$. (*I*) The viral inhibition of Rac1 activity recovers memory from amnesia [$n_{Ctrl+SAL(1\ h)} = 6$, $n_{Ctrl+SAL(24\ h)} = 12$, $n_{Ctrl+ANI(1\ h)} = 11$, $n_{Ctrl+ANI(24\ h)} = 10$, $n_{Rac1\text{-}DN+ANI(1\ h)} = 5$, $n_{Rac1\text{-}DN+ANI(24\ h)} = 16$]. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$; from unpaired *t* test (*C, F, and H*) and two-way ANOVA with Bonferroni test (*I*). Data are presented as means ± SEM.

bilaterally injected with ANI through the cannula (*SI Appendix*, Fig. S7*B*) immediately after the five-trial CFC (Fig. 3*G*). We found that ANI injection increased Rac1 activity 24 h after CFC in the control, but failed to increase Rac1 activity in Rac1-DN mice (Fig. 3*H* and *SI Appendix*, Fig. S8*A*). Accordingly, protein synthesis inhibition–induced amnesia was also prevented in Rac1-DN mice (Fig. 3*I*). Thus, as soon as Rac1 activity is unable to be evoked by CFC, protein synthesis inhibition fails to cause retrograde amnesia for contextual fear memory.

We also showed that ANI injection led to a further increase in Rac1 activity in response to single-shock CFC (*SI Appendix*, Fig. S9 *A–C*). To exclude the possibility of direct ANI stimulation of Rac1 activity, we assayed the effects of ANI on Rac1 activity without coupling to conditioning. Rac1 activity remained unaffected in mice subjected to either context-only or shock-only experience with injection of ANI (*SI Appendix*, Fig. S10).

Thus, the data presented support a role for Rac1-dependent forgetting in mediating ANI-induced reversible amnesia. Such observations led us to pursue a hypothesis that SS-dependent activation of Rac1 activity drives latent engrams to silence, while SR-dependent suppression of Rac1 activity switches silent engrams to latent engrams.

Acute Social Experiences Switch States of Memory Engrams by Regulating Hippocampal Rac1 Activity. The hypothesis elaborated above leads to three testable predictions. First, SR experience leads to suppression of Rac1 activity. Indeed, we found that a 10-min SR treatment was sufficient to reduce protein synthesis inhibitor–induced hyperactive Rac1 assayed 24 h after CFC (Fig. 4*H* and *SI Appendix*, Fig. S11*A*).

Second, direct inhibition of Rac1 activity through pharmacological treatment should mimic SR-mediated rescue effects on amnesic memory at the behavioral and engram-cell levels. We suppressed Rac1 activity by injecting the Rac1 inhibitor Ehop016 (i.p.) 30 min before testing the 24-h memory (Fig. 4*B*). Such treatment suppressed ANI-injection–elevated Rac1 activity (*SI Appendix*, Fig. S12) and rescued contextual fear memory from protein synthesis inhibition–induced amnesia (Fig. 4*C*). Consistent with the effect of SR (Fig. 1*B*), this rescue effect was also temporary, as memory dropped back 24 h later

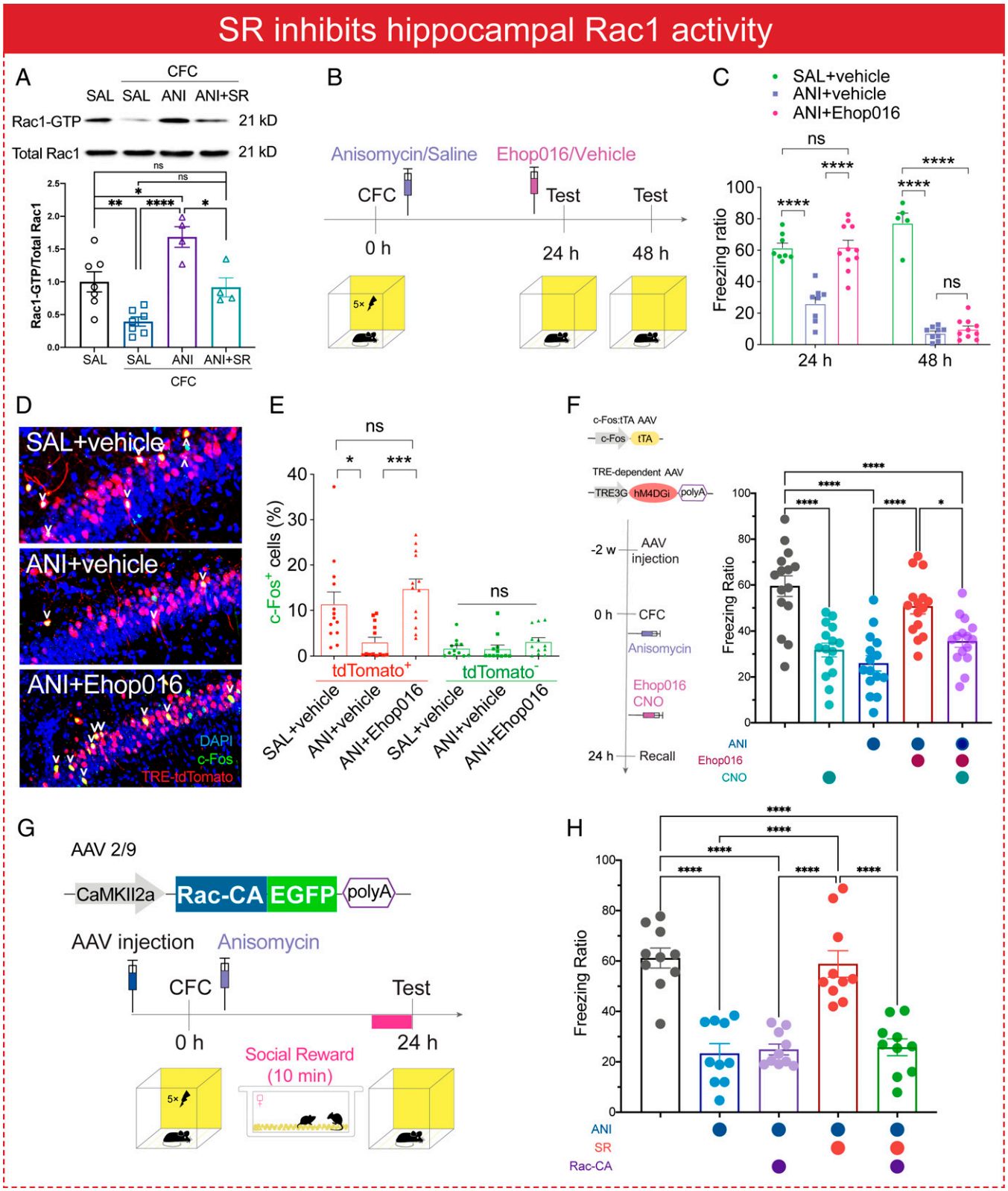

Fig. 4. Social reward switches silent memory engram to a latent state via inhibiting hippocampal Rac1 activity. (*A*) Immunoblotting and data showing Rac1 activity (Rac1-GTP) and total Rac1 levels in hippocampus (normalization of the value of Rac1-GFP/total Rac1: (*A*) Normalized to the SAL group at various retention intervals (n_{Naive} = 7, n_{SAL} = 7, n_{ANI} = 4, n_{ANI+SR} = 4). (*B*) Experimental schedule. (*C*) The effect of Ehop016 on contextual fear memory [$n_{SAL+vehicle(24\ h)}$ = 8, $n_{SAL+vehicle(48\ h)}$ = 5, $n_{ANI+vehicle(24\ h)}$ = 8, $n_{ANI+vehicle(48\ h)}$ = 9, $n_{ANI+Ehop016(24\ h)}$ = 11, $n_{ANI+Ehop016(48\ h)}$ = 10]. (*D*) Coronal section of CA1 engram cells (TRE-tdTomato) labeling with anti–c-fos (green). (Scale bar, 50 μm.) Arrowheads indicate double positive. (*E*) Percentages of c-Fos$^+$ cells in tdTomato$^+$ and tdTomato$^-$ cells in CA1 (12 to 13 slices from five mice for each group). (*F*) Strategy of chemogenetic inhibition of engram cells; experimental schedule; the effect of chemogenetic inhibition of engram cells on the rescue of amnesia by Ehop016. (*G*) Strategy of Rac1-CA expression and experimental schedule. (*H*) The effect of social reward on silenced memory is abolished in mice with the expression of Rac1-CA. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$; from one-way ANOVA with Tukey's test (*A*, *E*, *F*, *and H*) and two-way ANOVA with Bonferroni test (*C*). Data are presented as means ± SEM.

after the Ehop016 injection (Fig. 4*C*, see histograms over 48 h). We next examined the engram reactivation ratio in the dorsal CA1. We found that such pharmacological inhibition of Rac1 activity reversed the decreased reactivation ratio of engram cells resulting from protein synthesis inhibition (Fig. 4 *D* and *E* and *SI Appendix*, Fig. S3*C*), in a pattern consistent with the effects of SR (Fig. 1 *E* and *F*). In addition, we found that Ehop016 treatment also rescued CMX-induced silenced memory (*SI Appendix*, Fig. S13) and ANI-induced silenced memory with single-shock CFC (*SI Appendix*, Fig. S11 *B* and *C*). To test whether this Rac1 activity-dependent effect relied on the function of engram cells, we did a loss-of-function experiment in engram cells by expressing the chemogenetic inhibition tool hM4DGi (human M4 DREADD Gi) or tetanus toxin light chain (TeTX). We found that the inhibition of Rac1 had no effect on silenced memory with inhibiting engram-cell activity (Fig. 4*F*) or block the synaptic output of engram cells (*SI Appendix*, Fig. S14). Thus, direct inhibition of Rac1 activity in the hippocampus mimics the effects of SR treatment in converting silenced engrams to the latent state. To further validate that the inhibition of hippocampal activity underlies the effect of SR, we next tested whether SR still can rescue anisomycin-induced silenced memory when hippocampal Rac1 activity is elevated. By expressing constitutively active Rac1 (Rac1-CA) (33) in CA1, we found that the effect of SR on silenced memory was abolished in mice with expressing Rac1-CA. Together, our results indicate that SR switches silent memory engrams to the latent state via inhibiting hippocampal Rac1 activity.

The third prediction derived from the hypothesis of Rac1 activity–mediated switching of engram states would suggest that SS treatment would increase Rac1 activity to silent engrams. We assayed hippocampal Rac1 activity in mice subjected to 10 min of SS treatment 24 h after a five-trial CFC. Immunoblotting showed that SS treatment indeed induced an increase in hippocampal Rac1 activity (Fig. 5*A*). To validate the functional significance of this SS-induced increase, we showed that pharmacological inhibition of Rac1 activity through injection of Ehop016 (i.p.) (Fig. 5*B*) prevented SS-induced memory disruption (Fig. 5*C*).

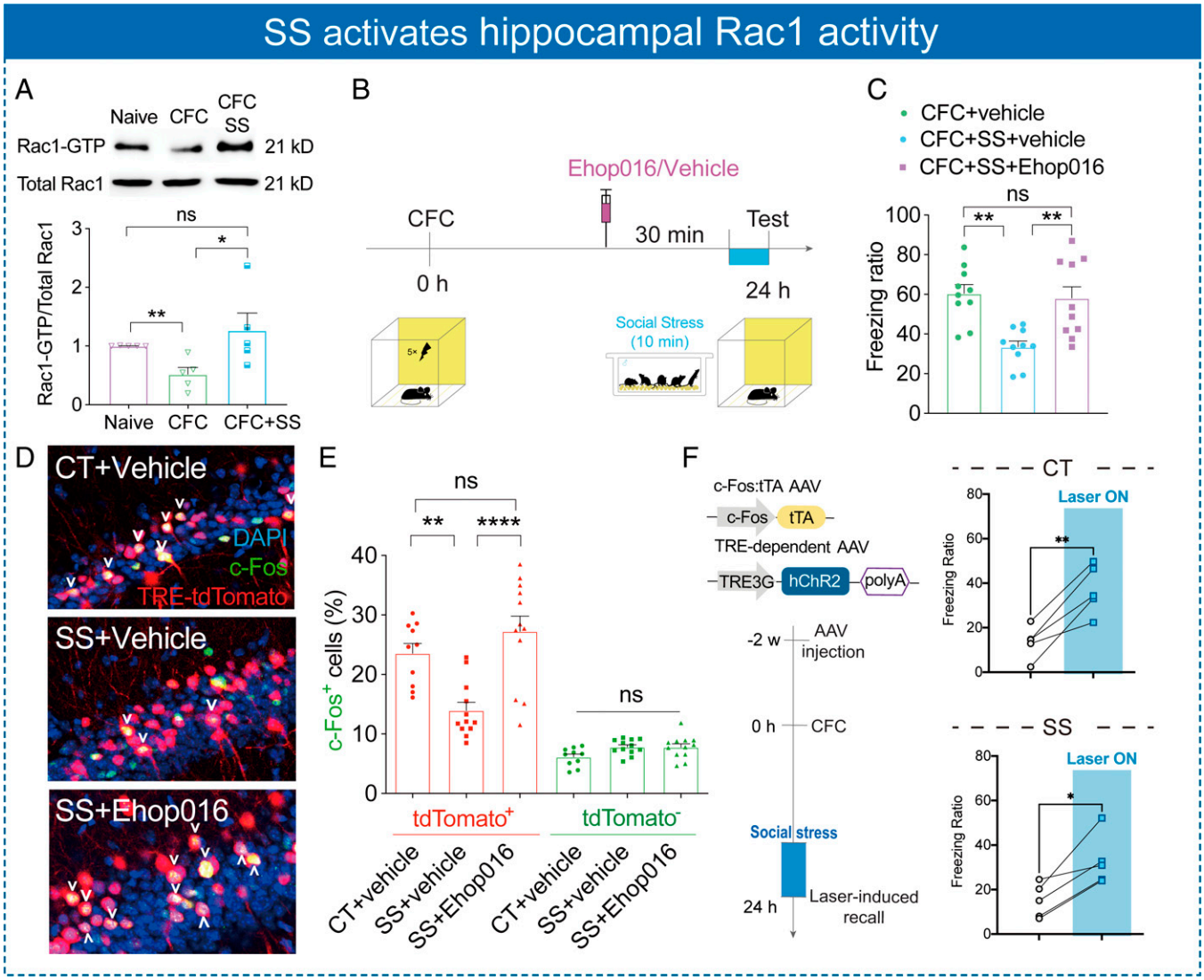

Fig. 5. Social stress silences memory engram via activating hippocampal Rac1 activity. (*A*) Immunoblotting and data showing Rac1 activity (Rac1-GTP) and total Rac1 levels in hippocampus (normalization of the value of Rac1-GFP/total Rac1) (*A*) Normalized to the naive group at various retention intervals (n_{Naive} = 7, n_{CFC} = 7, n_{CFC+SS} = 7). (*B*) Experimental schedule. (*C*) The effects of Ehop016 on contextual fear memory (n_{CFC} = 10, $n_{CFC+SS+vehicle}$ = 10, $n_{CFC+SS+Ehop016}$ = 10). (*D*) Coronal section of CA1 engram cells (TRE-tdTomato) labeling with anti–c-fos (green). (Scale bar, 50 μm.) Arrowheads indicate double positive. (*E*) Percentages of c-Fos$^+$ cells in tdTomato$^+$ and tdTomato$^-$ cells in CA1 (10 to 12 slices from five mice for each group). (*F*) Strategy of ChR2 expression in engram cells; experimental schedule; optogenetic activation of engram cells induces memory retrieval in mice with or without immediate SS in a novel context. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$; from paired *t* test (*F*) and one-way ANOVA with Tukey's test (*A*, *C*, and *E*). Data are presented as means ± SEM.

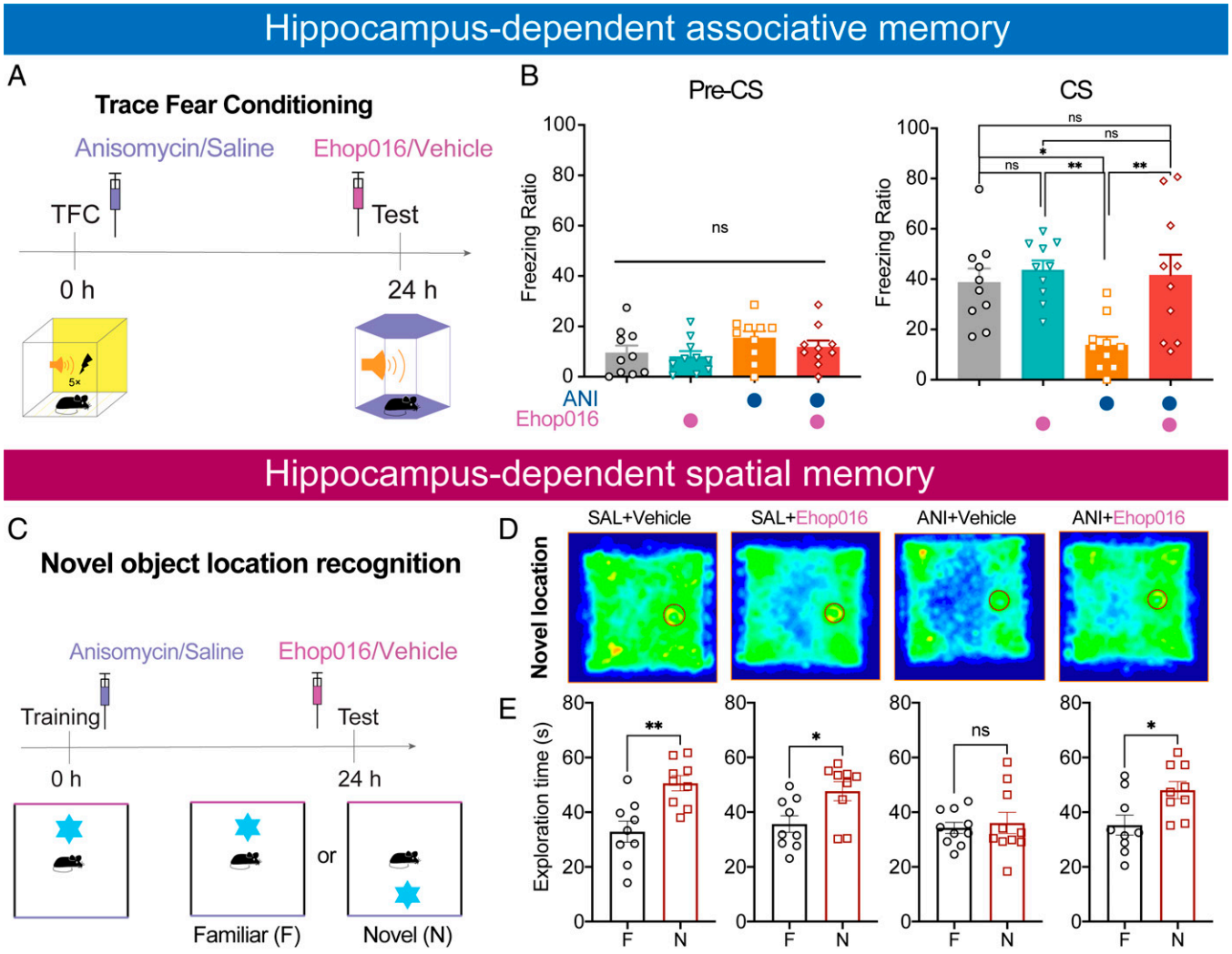

Fig. 6. Recovery of multiple types of hippocampus-dependent memories from amnesia. (*A* and *C*) Experimental schedule. (*B*) The levels of trace fear memory (Each group, $n = 10$). (*D* and *E*) Average heatmap showing exploration time for familiar (F) or novel (N) locations (*Left* or *Right*, respectively). Black circles represent object location (each group, $n = 9$). ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$; from unpaired *t* test (*E*) and one-way ANOVA with Tukey's test (*B*). Data are presented as means ± SEM.

We confirmed the role of SS-activated Rac1 activity in switching the latent memory engram to a silent engram by showing that an SS-triggered decrease in the engram-cell reactivation ratio could be rescued by inhibiting Rac1 activity (Fig. 5 *D* and *E*). Furthermore, by optogenetically activating engram cells in CA1 after SS, we found that this activation could both induce memory retrieval in mice with immediate SS or not in a novel context (34), which further supports that these engram cells are silent engram cells.

Thus, the data presented above confirmed all three predictions. Such strong evidence leads us to suggest that for contextual fear memory, SR treatment switches silent engrams to latent engrams through the suppression of Rac1 activity, while SS treatment switches latent engrams to silent engrams through elevations in Rac1 activity.

Recovery of Silent Memory Engrams in Multiple Hippocampus-Dependent Tasks through the Inhibition of Rac1 Activity. To gain a further understanding of the physiological significance of the identified novel Rac1-dependent mechanism that mediates switching between engram states, we expanded experiments into other hippocampus-dependent tasks, including trace fear conditioning and novel object location (NOL) recognition. In the trace fear conditioning task, a mouse was subject to a five-trial conditioning in which a tone (1,200 Hz) is paired with an electric shock, and a memory test is performed in a novel context (Fig. 6*A* and *Materials and Methods*). We found that ANI injections immediately after conditioning disrupted 24-h trace memory, while a single injection of Ehop016 before the memory test recovered the ANI-induced amnesic memory (Fig. 6*B*, with conditioned stimulation [CS]). Without the CS, there were no significant differences in freezing levels among the three groups of mice (Fig. 6*B*, without CS or pre-CS). Thus, inhibition of Rac1 activity was sufficient to reverse protein synthesis inhibition–induced amnesia in trace memory. In the novel object location task (Fig. 6*C*), the ANI-injected mice showed 24-h spatial memory defects in the novel object location test. Similarly, the rescue effect mediated by Ehop016 injection was observed in amnesic mice (Fig. 6 *D* and *E*). Thus, the presented data support the idea that inhibition of Rac1 activity leads to switching of silent engrams to latent engrams in multiple hippocampus-dependent tasks.

Discussion

The current work investigates whether and how emotional states evoked by acute social interactions regulate memory engrams. This study leads to two major findings: First, social interaction–evoked emotions can bidirectionally switch the

storage states of memory engrams, as SR turns a silent engram into the latent state, while SS converts a latent engram to silent in CA1 neurons of the hippocampus. Second, SR suppresses, and SS evokes the hippocampal Rac1 activity, while the effects of such Rac1 regulation are consistent with respective impacts of SR and SS on engrams. Taken together, the current work provides a neurobiological mechanism for how acute emotional changes can transiently affect cognitive processing by switching states of memory engrams stored in the hippocampus.

A major feature of the memory engram in the hippocampus is revealed as that memory can be stored not only as active or latent engrams available for natural recall but also as silent engrams that can be retrieved only through artificial stimulation (16, 21, 35). Specifically, in protein synthesis inhibition–treated mice (19, 21) or the Alzheimer's disease model (35), memory is stored as a silent engram, while via some artificial approaches (22, 35), such silenced memory engrams can be converted to the latent state. However, whether and how multiple engram states, and silent engrams, in particular, are present and switchable between engram states in normal physiological or psychological conditions remain elusive. We demonstrated that acute SR treatment recovers protein synthesis inhibition–induced silenced memory, while SS temporarily disrupts memory retrieval. These transient alterations in memory retrievability are associated with increases or decreases, respectively, in the ratio of reactivated engram cells to learning-activated total engram cells after ANI injection (Fig. 1). Our data suggest that such diverse states of memory engram confer the dynamics and flexibility to memory, allowing animals to retrieve prior knowledge according to the current conditions.

For molecular mechanisms underlying the switching of engram states, we presented multiple lines of evidence supporting the idea that such switching is mediated through emotion-dependent regulation of Rac1 activity. First, hippocampal Rac1 activity correlates well with memory engram states, as Rac1 activity is inhibited after CFC and dramatically elevated with protein synthesis inhibition. Furthermore, the inhibition of Rac1 activity can antagonize the effect of protein synthesis inhibition (Fig. 2). In addition, silenced memory engrams and elevated hippocampal Rac1 activity are both observed in Alzheimer's disease model mice (32, 35), supporting our conclusion. Second, SR suppresses and SS evokes the regulation of hippocampal Rac1 activity to achieve their respective impact (Figs. 4 and 5), and the overexpression of constitutively active Rac1 blocks the effect of SR while the pharmacological inhibition of Rac1 (Fig. 4) prevented SS switching of a latent engram into the silent state (Fig. 5). Supportively, previous studies have reported that Rac1 activity responses to social stress and mediates the regulation of related behaviors (36, 37). Third, chemogenetically inhibiting activity (Fig. 4*F*) or blocking synaptic outputs (*SI Appendix*, Fig. S14) of engram cells made pharmacological inhibition of Rac1 activity fail to recover protein synthesis inhibition–induced silenced memory, suggesting observed Rac1 effects act through engram cells. Fourth, such Rac1-dependent regulation of engram states is not confined to CFC memory but is also observed in other hippocampus-dependent memories (Fig. 6).

In light of the anisomycin-induced impairment of synaptic connection underlying the silence of memory engrams (19, 21, 22), and given that Rac1 is crucial for the dynamics of synaptic connectivity (38–40), this Rac1-dependent switching between latent and silent engram states may recruit the downstream of Rac1 to regulate synaptic structure in engram cells. A recent study reported the possible downstream of Rac1 for regulating memory: Rac1 activates a WASP family protein SCAR/WAVE to cause active forgetting of memory, and Rac1/SCAR may function with formin diaphanous, a nucleator that facilitates linear actin polymerization, to regulate memory forgetting by modifying synaptic connectivity (41). It provides a possible explanation for how Rac-1 mediates such emotion-mediated switching of engram states.

Although the involvement of hippocampal Rac1 activity in emotion-mediated regulation of memory is strongly supported via the data presented, we could not rule out an involvement of other signaling pathways in switching memory engram states, considering that learning-evoked protein synthesis induces expression of diverse molecules (42). Specifically, brain-derived neurotrophic factor (BDNF) is capable of restoring amnesic memory similar to Rac1 effects (43), and the activation of PAK-1 also can restore the silenced memory engram (21). However, it remains unclear whether these signaling molecules act through pathways independent of Rac1.

Thus, the current study develops two emotional paradigms to switch engram reversibly from a silent state to a latent state and provides a potential molecular mechanism for understanding how the current emotional state affects memory accessibility. Multiple lines of evidence are presented to support the idea that hippocampal Rac1 activity plays a crucial role in mediating the emotion-dependent switching of engram states.

Materials and Methods

Animals. C57BL/6J (age 3 to 4 mo, male) were purchased from the Vital River Laboratory (Animal Technology) and were housed in groups under standard conditions according to the Tsinghua University animal facility. Mice were maintained on a 12-h light/dark cycle and tested during the light phase of the cycle. All animal work complied with ethical regulations for animal testing and research, and was done in accordance with Institutional Animal Care and Use Committee approval by Tsinghua University and followed all Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

Drugs. Ehop016 (Shanghai Sun-shine Chemical Technology Co., Ltd.) was dissolved in a solution containing 1% dimethylsulfoxide (DMSO)/30% polyethylene glycol 300/1% Tween-80. Mice were intraperitoneally injected with 20 mg/kg of Ehop016 solution or an equivalent volume of saline 30 min before the test. At the dose used, Ehop016 has high efficiency to block Rac1 activity. Anisomycin (Selleck, Cat. No. S7409) was dissolved in 0.9% saline and the pH was adjusted with 1 N HCl to 7.0 to 7.4. Mice received a single 150 mg/kg of anisomycin injection immediately or multiple 50 mg/kg of anisomycin injections immediately, 2, 4, and 6 h after acquisition. A total of 150 mg/kg anisomycin was shown to effectively inhibit cerebral protein synthesis in mice (~96%). The multiple injections of anisomycin should sustain protein synthesis inhibition at levels >90% until 2 h after the final injection, resulting in strong protein synthesis inhibition for 8 h in mice that received multiple injections. For the CXM experiment, 9 mg/kg CXM, or equivalent SAL, was delivered subcutaneously immediately after training.

Surgery and Viral Injections. Mice were anesthetized with 0.2% sodium pentobarbital (5 mL/kg) and fixed to a stereotaxic frame. Their body temperature was kept at 36 °C by a heating pad and their skull was exposed. Bilateral craniotomies were performed using a 0.5-mm diameter micromotor drill. The injection of the virus was performed using a 10-mL NanoFil syringe under the control of the UMP3 and Micro4 system (WPI), with a speed of 50 nL/min. The dorsal CA1 injections were bilaterally targeted to −2.0 mm anterior-posterior (AP), 1.5 mm medial-lateral (ML), and −1.5 mm dorsal-ventral (DV). The viral volumes of the AAV-CaMKIIα-Rac1-DN-EGFP, and AAV-CaMKIIα-EGFP (as the control group) were 400 nL for the CA1 excitatory neurons. For labeling of the CA1 engram cells, a virus mixture AAV2/9-c-fos:tTA (300 nL of tTA) and 300 nL of TRE-tdTomato into the CA1 (−2.2 mm AP, ±1.7 mm ML, and −1.6 mm DV). For the gain- and loss-of-function experiments in engram cells, AAV2/9-TRE:-hChR2-eYFP, AAV2/9-TRE:-TeTX-P2A-eGFP, and AAV2/9-TRE:-hM4DGi-mCherry were used. All viral vectors were aliquoted and stored at −80 °C until use. After the injections, the needle remained in place for 10 min before slowly being withdrawn and the

wound was sutured. After surgery, animals were allowed to recover for 2 wk prior to the performance of all subsequent experiments. All the AAVs were purchased from BrainVTA and Vigene Biosciences.

Cannula Placements and Drug Infusion. Mice were implanted under 0.2% sodium pentobarbital (5 mL/kg) with guide cannula (RWD Life Science, Cat. No. 62522) in the dorsal CA1 region of the hippocampus in accordance with coordinates 2.0 mm AP, ±1.5 mm ML, and −2.0 mm DV. The cannulae were fixed to the skull with dental acrylic. Immediately after the training, WPI injectors were lowered through the guide cannula and mice received 0.25 μL of anisomycin (100 μg/μL) or saline per side at the rate of 0.1 μL/min.

Fear Conditioning. The fear conditioning test was conducted using the HABITEST Modular Behavioral Test System. A Coulbourn Habitest chamber (27 cm × 28 cm × 30.5 cm) had a stainless-steel rod floor, which was connected to a shock generator in a sound-attenuating box. In single-shock CFC sessions, mice were individually placed in the conditioning and they freely explored the area for 3 min. Then, mice were exposed to one footshock (2 s, 0.8 mA) and returned to their home cage 30 s after. In the five-shock contextual fear conditioning task, the footshock (2 s, 0.8 mA) was delivered at 180 s, 240 s, 300 s, 360 s, and 420 s. Mice remained in the conditioning chamber for a total of 450 s. During testing, mice were placed back in the conditioning chamber for 4 min. For trace fear conditioning, the mice were placed in the chamber and allowed to explore for 3 min. Then, a 20-s tone (80 dB, 1,200 Hz) was administered five times, followed by a 2-s 0.6 mA shock, with a 4-min interval between repetitions. The mice were removed 30 s after the last shock, and the tone and shock were separated by a 20-s interval. For the trace fear memory test, the mice were placed in a new environment with a 3-min period followed by three 20-s tones (CS), with a 220-s interval between repetitions. For the context-only group, mice were placed into the fear conditioning chamber for 3 min with no shock being delivered and then put back into their homecages. For the shock-only group, mice were exposed to an immediate electric shock (2 s, 0.8 mA) and then put back into their homecages. For all experiments, freezing levels of mice were accessed by an automated motion detection software (Freezeframe software; Actimetrics). Threshold values of motion index were set to 10 in all the experiments.

Social Reward/Social Stress. Acute social reward/stress treatment was as follows: For social reward treatment used throughout the whole study, we placed two female mice, aged between 4 and 8 wk, in a cage (width [W], 16 cm; diameter [D], 28 cm; and height [H], 13 cm) with soft packing beforehand for 5 to 10 min to acclimate the mice to the new environment. Then, we placed the male mouse to be tested in contextual fear conditioning into the cage for 10 min. For social stress treatment used throughout the whole study, we placed the male mouse to be tested into a home cage containing five male mice for 10 min. For social reward treatment using juveniles, we placed two juvenile mice, aged 4 wk, in the cage (W, 16 cm; D, 28 cm; and H, 13 cm) with soft packing beforehand for 5 to 10 min to acclimate the mice to the new environment. Then, we placed the male mouse to be tested into the cage for 10 min. For social stress induced by social defeat, we placed the male mouse to be tested into a home cage containing a retired male CD-1 breeder mouse (6 to 10 mo old) for 10 min.

Optogenetic Activation of Engram Cells. Immediately after the social stress, mice with expressing hChR2 in CA1 engram cells were placed in a context distinct from the CFC training context. ChR2 was stimulated at 20 Hz (10-ms pulse width) via a 474-nm laser (15 mW). Mice received four 2-min epochs. The first and third epochs were laser ON while the others were laser OFF. In this experiment, freeze counting was conducted manually and was double blind.

Novel Object Location. The NOL task consisted of a training phase and a testing phase. On the first day, mice were trained in a chamber (50 cm × 50 cm × 40 cm) with different patterns on the left and right walls. An object was placed near either patterned wall and mice were allowed to explore the chamber for 10 min. The location of the object was counterbalanced among each group. At 24 h after training, each group of mice was placed into the chamber with the object either in the familiar location or a novel location for 5 min. Exploration of the object was recorded using an animal behavior video tracking system (ANY-Maze) when the animal's nose was pointed at the object with a maximum distance of 2 cm from the object. The heatmap was plotted in the ANY-Maze software based on the average exploration time of each group of mice.

Western Blotting. Isolated hippocampi were homogenized in cell lysis buffer (Beyotime, Cat. No. P0013) with protease inhibitors. The protein concentration was determined using the BCA protein assay kit (Beyotime, Cat. No. P0012). For the detection of the levels of total-Rac1, 20 μg of homogenates was separated by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Pall Corporation, Cat. No. 66485). Membranes were blocked in milk solution (5% milk in tris-buffered saline (TBS) and 0.1% Tween 20) for 1 h at room temperature. Subsequently, membranes were individually incubated with primary antibodies against total-Rac1 (1:2,000, rabbit) overnight at 4 °C. All the horseradish peroxidase (HRP)-conjugated secondary antibodies were used at 1:2,000 dilutions for membrane incubation at room temperature for 1 h. For the relative level of the Rac1-GTP assay, GST-tagged PAK-PBD beads (Cytoskeleton, Cat. No. PAK02) were incubated with the lysate mix (300 μg) overnight at 4 °C. The beads were washed three times with lysis buffer at room temperature. Then, bound proteins were eluted with loading buffer (Beyotime, Cat. No. P0015B) and analyzed by Western blotting. Membranes were individually incubated with primary antibodies against Rac1-GTP (1:2,000, rabbit) overnight at 4 °C. Image quantification analysis in bands of the Western blots was calculated by ImageJ software (NIH).

Activity-Dependent Cell Labeling. Mice were bilaterally injected with a virus mixture (300 nL of $AAV_{2/9}$-c-fos:tTA) and 300 nL of the $AAV_{2/9}$-c-fos-tdTomato into the dorsal CA1. Mice were allowed to recover for 2 wk and raised on food containing 40 mg/kg doxycycline (Dox). In order to open a window of activity-dependent labeling for CFC, the mice were then taken off Dox for 24 to 30 h. After CFC, Dox diets were resumed immediately. For detection of the c-Fos immunoreactivities in the CA1, animals were perfused and their brains were fixed with 4% paraformaldehyde (PFA) after the exposure to recall cues (the conditioned context). Coronal slices were incubated with anti–c-Fos antibody (Cell Signaling Technology, Cat. No. 2250). The images of the immunohistochemistry were captured on a Zeiss LSM 710 confocal microscope.

Cell Counting. When we evaluated the reactivation of engram cells by natural recall cues, 20 coronal hippocampal sections were used to count the number of $tdTomato^+$ cells, $c\text{-}Fos^+$ cells, and $DAPI^+$ cells. The numbers of $tdTomato^+$ cells, $c\text{-}Fos^+$ cells, and $DAPI^+$ cells in the region of CA1 were counted by utilizing the imaging analysis function of the Zeiss software (Zen blue 2.3). The quantification of the number of $tdTomato^+$ cells was performed by setting the threshold manually from 1,000 to 4,096. The quantification of the number of $c\text{-}Fos^+$ cells was performed by setting the threshold manually from 692 to 4,096. The quantification of the number of cells with DAPI was performed by setting the threshold manually from 616 to 4,096. The ratios of $c\text{-}Fos^+$ $tdTomato^+$/$tdTomato^+$ and the ratios of $c\text{-}Fos^+$ $tdTomato^-$/$tdTomato^-$ were calculated.

Statistics. Statistical analyses were performed in GraphPad Prism. All data were analyzed with an unpaired *t* test, one-way ANOVA, or two-way ANOVA where appropriate. The data are shown as the mean ± SEM and ns indicates nonsignificance ($P > 0.05$). The significant levels were set to $P = 0.05$. Significant for comparison is as follows: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. The sample sizes (*n*), specific statistical tests used, main effects, and the *P* values for each experiment can be found in *SI Appendix*, Table S1.

Data Availability. All study data are included in the article and *SI Appendix*.

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