Optogenetic reactivation of memory ensembles in the retrosplenial cortex induces systems consolidation

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The neural circuits underlying memory change over prolonged periods after learning, in a process known as systems consolidation. Postlearning spontaneous reactivation of memory-related neural ensembles is thought to mediate this process, although a causal link has not been established. Here we test this hypothesis in mice by using optogenetics to selectively reactivate neural ensembles representing a contextual fear memory (sometimes referred to as engram neurons). High-frequency stimulation of these ensembles in the retrosplenial cortex 1 day after learning produced a recent memory with features normally observed in consolidated remote memories, including higher engagement of neocortical areas during retrieval, contextual generalization, and decreased hippocampal dependence. Moreover, this effect was only present if memory ensembles were reactivated during sleep or light anesthesia. These results provide direct support for postlearning memory ensemble reactivation as a mechanism of systems consolidation, and show that this process can be accelerated by ensemble reactivation in an unconscious state.

engram | memory consolidation | retrosplenial cortex | fear conditioning | replay

The ability to encode and retrieve episodic memories requires coordinated activity in diverse brain areas, including the thalamus, neocortex, and areas of the medial-temporal lobe such as the hippocampus (HPC) (1–3). At the time of learning, synaptic plasticity is thought to occur in a subset of neurons that are activated during the experience and become part of the neural ensemble representing the specific memory, sometimes referred to as the memory engram (4). These changes occur rapidly with memory encoding, and are essential for the initial formation and maintenance of memory (5, 6). As time passes, memory ensembles throughout the brain are further stabilized and modified through a process known as systems memory consolidation, which is thought to be necessary for the maintenance, integration, and correct categorization of new information (7, 8). This process is usually slow (months to years in humans and weeks to months in rodents) and changes the relative contribution of different brain areas for memory retrieval. Studies from both humans and rodents show that the hippocampus is preferentially engaged during learning and recent memory retrieval, whereas neocortical areas are more active when a remote memory is retrieved (9–11). In addition, some neocortical areas involved in remote memory are not necessary for recent memory retrieval (9, 12), whereas the hippocampus is generally dispensable for remote memory retrieval (13–16), although some recent studies have challenged this idea (12, 17). Interestingly, these broad changes at the neural circuit level are often accompanied by changes in the quality of memory. For example, humans tend to lose details of episodic memories as time passes (18), and rodents are unable to discriminate between two different contexts in a remote retrieval trial in the context fear conditioning (CFC) paradigm (19). The mechanism underlying these changes is unclear; however, some models propose that spontaneous postlearning reactivation of the neural ensembles involved in memory encoding gradually promotes neocortical ensemble maturation and systems consolidation through activity-dependent synaptic plasticity within these circuits (7, 8, 20). In support of this hypothesis, correlative studies in both humans and animal models have demonstrated coherent reactivation of learning-related neural activity during offline brain states (i.e., sleep and quiet awake states) (21–23), and disruption of high-frequency rhythmic activity associated with this ensemble reactivation in rodents impairs performance in spatial tasks (24, 25). However, there is no evidence that reactivation of neural ensembles representing a memory is directly involved in systems consolidation. Demonstration of such causal relation requires either selective inhibition (loss of function) or activation (gain of function) of neural ensembles representing a specific memory to evaluate its effect on the consolidation process.

In this study, we adopted a gain-of-function strategy to test the role of the reactivation of neocortical memory ensembles in systems consolidation. We used a *cfos*-based genetic tagging system (cfos-tTA/tetO-ChEF transgenic mouse) to express a channelrhodopsin variant (ChEF) selectively in neurons naturally activated during CFC (26), and subsequently stimulated these ensembles in the retrosplenial cortex (RSC), using high-frequency optogenetic stimulation (Fig. 1). The RSC is well suited to contribute to the consolidation process, as it is connected to both the hippocampus

Significance

This study examines a question in memory research that has been extant since the observation of temporally graded retrograde amnesia in patient HM after temporal lobe resection; namely, how does the circuit structure underlying memory change over prolonged periods after initial learning, such that recent memories require the hippocampus whereas older remote memories do not? We use optogenetic reactivation of the neurons active naturally during initial learning (engram neurons) to show that postlearning neural replay can produce this shift in memory. Interestingly, this was only observed in sleeping, not awake, mice. This is consistent with a model in which natural activity in memory ensemble during offline periods results in the circuit change to remote memory.

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The authors declare no conflict of interest.

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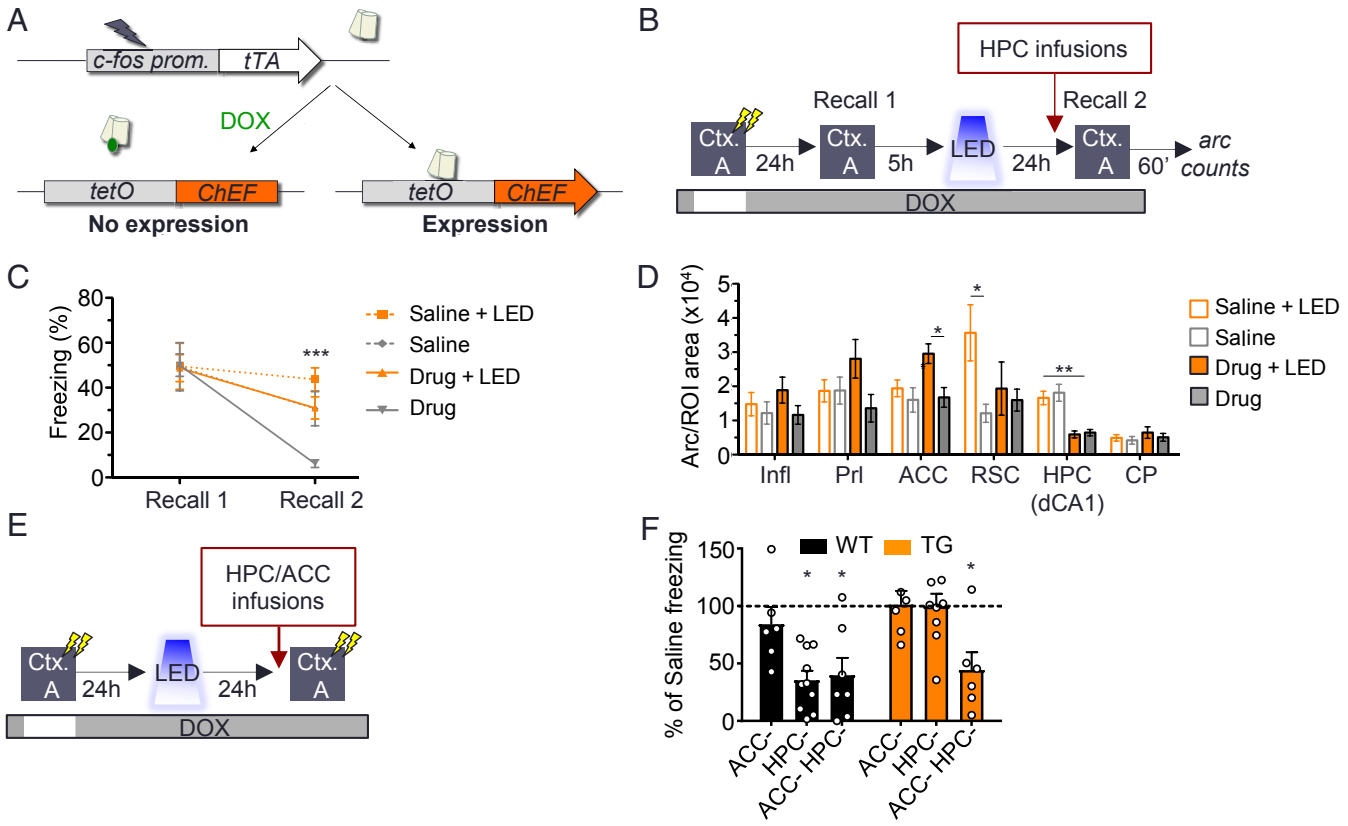

Fig. 1. Optogenetic reactivation of RSC memory ensembles produces a recent memory with characteristics of a remote memory. (*A*) Schematic of the *cfos-tTA/tetO-ChEF* double-transgenic mouse line. (*B*) Experimental protocol used in RSC ensemble reactivation and HPC inactivation. (*C*) Freezing levels of TG animals before (recall 1, 24 h after training) and after (recall 2, 48 h after training) light-emitting diode (LED) stimulation and HPC infusions. (saline+LED, $n = 6$; saline, $n = 6$; drug + LED, $n = 9$; drug, $n = 9$). (*D*) *arc*-positive cells in selected brain areas after recall 2 (saline+LED, $n = 6$; saline, $n = 5$; drug + LED, $n = 5$; drug, $n = 6$). (*E*) Experimental protocol used in RSC ensemble reactivation and HPC/ACC inactivation. (*F*) Freezing levels of WT and TG animals after LED stimulation and HPC or ACC drug infusions (WT/ACC$^-$, $n = 6$; WT/HPC$^-$, $n = 10$; WT/ACC$^-$/HPC$^-$, $n = 7$; TG/ACC$^-$, $n = 6$; TG/HPC$^-$, $n = 9$; TG/ACC$^-$/HPC$^-$, $n = 6$). Drug, CNQX+TTX; Infl, infralimbic cortex; Prl, prelimbic cortex; ACC, anterior cingulate cortex; HPC, hippocampus; CP, caudate putamen. In all panels, bars represent mean ± SEM. *$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

and neocortical areas required for remote memory, and is necessary for both recent and remote CFC memory retrieval (27), and we have previously shown that the cfos-positive neurons activated with learning carry a component of the original CFC memory trace (26). We found that posttraining stimulation of the RSC neural ensembles activated during CFC learning generated a recent memory that displayed several features of consolidated remote CFC memories, including decreased hippocampal dependence, context generalization, and greater engagement of neocortical areas during retrieval, suggesting that this type of activity is able to induce physiological changes similar to those observed during natural systems consolidation. Moreover, these changes were only observed when ensemble reactivation was performed during light anesthesia or natural sleep, but not with reactivation during active awake states.

Results

To test the hypothesis that postlearning reactivation of neurons representing a memory is involved in systems consolidation, we stimulated RSC neural ensembles representing a recent CFC experience (1 d after conditioning) with a high-frequency optogenetic stimulation protocol known to induce long-term potentiation in vivo (28). Transgenic cfos-tTA/tetO-ChEF mice (TG) were fear-conditioned in the absence of doxycycline to allow ChEF expression in RSC neurons that were activated during learning. After CFC, animals were returned to their home cage and fed doxycycline-containing food for the remainder of the experiment to prevent any further genetic labeling. The following day, animals were exposed to the training context for a brief recall trial and distributed into four groups matched by freezing levels (Fig. 1*B*). To achieve a stable "offline" brain state during stimulation of memory ensembles, two groups were lightly sedated with isoflurane anesthesia (*SI Appendix*, Fig. S1) while optogenetic stimulation was delivered to the RSC. The other two groups underwent the same procedure, but without optogenetic stimulation. Twenty-four hours after stimulation, two groups (one from each light condition) received intrahippocampal infusions of a mixture of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and tetrodotoxin (TTX) to block neuronal activity in this brain region, and the other two groups received saline infusions. Interestingly, we observed that animals that had received optogenetic stimulation did not show memory impairment induced by hippocampal inactivation (Fig. 1*C*). This result indicates that the optogenetic stimulation generated a recent memory that is not dependent on HPC activity, similar to what is observed during retrieval of remote, consolidated CFC memories (14, 15) (*SI Appendix*, Fig. S2). Moreover, this effect was only observed when memory-specific neural ensembles were stimulated, suggesting that the observed effect is not a general consequence of RSC stimulation (*SI Appendix*, Fig. S3). To gain further insight into which brain areas could be supporting memory retrieval in the absence of a functional hippocampus, we killed all animals from Fig. 1*C* 60 min after memory retrieval and immunostained for the expression of the immediate early gene *arc*. We observed that different groups of TG animals

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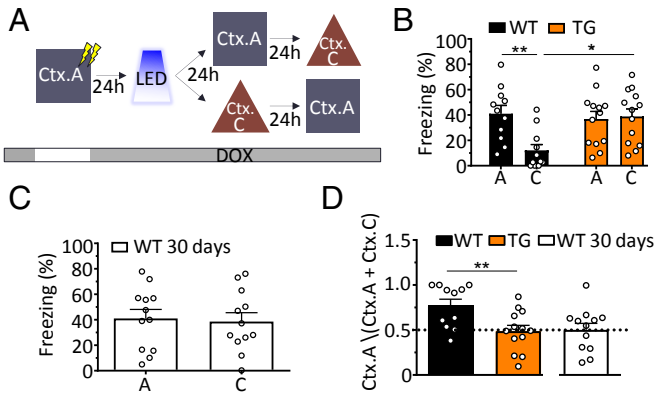

Fig. 2. Optogenetic reactivation of RSC memory ensembles produces context generalization. (*A*) Experimental protocol used to test memory generalization. (*B*) Freezing levels of animals in training context (ctxA) or a novel context (ctxC) after RSC stimulation (TG, $n = 13$; WT, $n = 11$). (*C*) Freezing levels of WT animals 30 d after CFC in ctxA or ctxC ($n = 12$). (*D*) Discrimination indexes of stimulated WT and TG mice after optogenetic stimulation induced consolidation and natural consolidation (WT, 30 d). In all panels, bars represent mean ± SEM. $*P < 0.05$; $**P < 0.01$.

that received optogenetic stimulation had increased brain activity in frontal cortical areas that previous studies have shown are recruited during the retrieval of remote memories (9, 10, 29) (Fig. 1*D*). Specifically, the anterior cingulate cortex (ACC) showed a significant increase in activity in stimulated animals that retrieved the memory in the absence of a functional HPC. In contrast, animals that had received optogenetic stimulation but had a functioning HPC (saline infusion group) failed to show increased activity in the ACC, but did show increased activity in the RSC during memory retrieval (Fig. 1*D* and *SI Appendix*, Fig. S4). These data indicate that high-frequency stimulation of RSC memory ensembles increases neocortical engagement without decreasing HPC participation during memory retrieval, and increases ACC activation only when the HPC is not functional, suggesting that the ACC is compensating for the lack of a functional HPC. We therefore investigated the individual contribution of these two brain regions for supporting memory retrieval after RSC optogenetic stimulation. We used a behavioral schedule similar to the one described in Fig. 1*B*, but this time animals were not matched by freezing levels on a prestimulation retrieval trial, and we used light-stimulated WT mice as controls (Fig. 1*E*). Again, we observed that HPC infusions of CNQX+TTX did not affect memory retrieval in TG animals that had received RSC optogenetic stimulation (Fig. 1*F* and *SI Appendix*, Fig. S5*B*). We then performed the same protocol, but infused one group of animals with CNQX+TTX in the ACC and another group in both the ACC and HPC before memory retrieval. Interestingly, memory impairment in TG animals was only observed when both areas were simultaneously disrupted before memory retrieval (Fig. 1*F* and *SI Appendix*, Fig. S5*C*). Together, these results suggest that reactivation of RSC memory ensembles generates a recent memory that is not affected by HPC disruption, engages more neocortical areas, and can be retrieved through a cortical circuit involving the ACC in the absence of a functional HPC.

We next asked whether this apparent circuit rearrangement induced by RSC optogenetic stimulation could have an effect on the quality of the CFC memory representation by examining context discrimination. We trained TG and WT mice in context A (ctxA) and stimulated the RSC using the same high-frequency optogenetic protocol during light anesthesia, as shown in Fig. 2*A*. Twenty-four hours after optogenetic stimulation, all animals were exposed to either ctxA or a similar context C (ctxC), followed by exposure to the opposite context the next day in a counterbalanced design. As expected, control WT animals could discriminate between the conditioned ctxA and the new ctxC (Fig. 2*B*). However, TG mice that underwent optogenetic stimulation showed a generalized fear response in both contexts, similar to WT animals following natural systems consolidation after CFC (Fig. 2 *C* and *D*) (19).

The results presented here indicate that high-frequency stimulation of RSC memory ensembles under isoflurane anesthesia is sufficient to induce apparent systems consolidation, as measured by the need of the hippocampus for memory retrieval, brain areas activated during memory retrieval, and context generalization. To investigate the existence of a possible contribution of isoflurane for the observed effect, we performed the same behavioral protocol but stimulated RSC ensembles during natural sleep (see *Methods* for details). Quantification of the number of wake episodes and total percentage of sleep time during RSC stimulation revealed no significant differences between WT and TG mice, indicating that RSC stimulation does not disturb the macrostructure of sleep in TG mice (*SI Appendix*, Fig. S6 *B* and *C*). One day after RSC stimulation, all mice were tested in the conditioned context with (CNQX+TTX group) or without (saline group) hippocampal inactivation. Similar to our results with isoflurane, we observed that TG animals that had received optogenetic stimulation during natural sleep were able to successfully retrieve the conditioned memory after HPC inactivation (Fig. 3*B*). The following day, mice that had received intrahippocampal infusions of saline solution before memory retrieval were tested in ctxC to evaluate the effect of RSC stimulation during sleep in context generalization. Similar to the results obtained in Fig. 2, TG mice generalized their conditioned response to ctxC, whereas WT mice were able to distinguish between these two contexts (*SI Appendix*, Fig. S6*D*). All animals were further tested for increased levels of anxiety on an elevated plus maze and in an open field arena. The results indicated no significant differences between WT and TG mice with respect to the time spent in the open arms of the elevated plus maze or the time spent in the center of the open arena, suggesting that the generalization results were not a consequence of increased levels

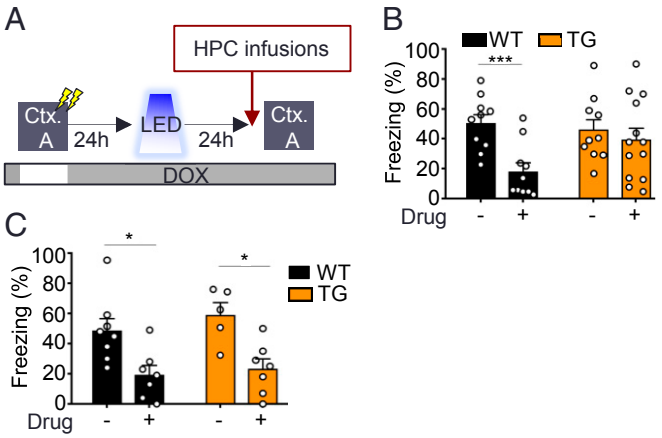

Fig. 3. The effect of optogenetic memory ensemble reactivation is dependent on brain state. (*A*) Experimental protocol used in RSC ensemble reactivation and HPC inactivation. (*B*) Freezing levels of WT and TG animals during retrieval trial after RSC ensemble reactivation during sleep and HPC infusions (WT/CNQX$^-$, $n = 10$; WT/CNQX$^+$, $n = 10$; TG/CNQX$^-$, $n = 10$; TG/CNQX$^+$, $n = 13$). (*C*) Freezing levels of WT and TG animals during retrieval trial after RSC ensemble reactivation during awake and HPC infusions (WT/CNQX$^-$, $n = 8$; WT/CNQX$^+$, $n = 7$; TG/CNQX$^-$, $n = 5$; TG/CNQX$^+$, $n = 7$). In all panels, bars represent mean ± SEM. Horizontal axis indicates groups that received hippocampal inactivation (CNQX+TTX) before memory retrieval. $*P < 0.05$; $***P < 0.001$.

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of anxiety in TG mice (*SI Appendix*, Fig. S6 *E* and *F*). Taken together, these observations suggest that the consolidation effect described here can be achieved by stimulating RSC memory ensembles during natural sleep and anesthesia.

The results presented here suggest that high-frequency stimulation of RSC ensembles during "offline" brain states is sufficient to drive apparent systems memory consolidation. To investigate whether this brain state is necessary for the observed effect, we designed an experiment to stimulate the same RSC ensembles during active awake periods. We performed the same protocol described in Fig. 3*A*, but this time delivered optogenetic stimulation while TG and WT mice explored a novel open arena. We observed no differences in immobility levels between both groups during awake stimulation (*SI Appendix*, Fig. S7*B*), suggesting this stimulation protocol is not sufficient to induce the apparent retrieval of the conditioned response in TG mice that has been previously observed when using lower stimulation frequencies (5 Hz) (26). Moreover, we observed that TG animals did not present any signs of increased locomotion or increased anxiety during high-frequency stimulation of RSC ensembles (*SI Appendix*, Fig. S7 *C* and *D*). The following day, TG and WT mice were tested in the conditioned context with (CNQX+TTX group) or without (saline group) hippocampal inactivation, as described earlier. Surprisingly, TG animals stimulated during active awake states were unable to successfully retrieve the CFC memory after HPC inactivation, suggesting that the RSC optogenetic stimulation protocol affects memory circuits differently, depending on the brain state at the time of stimulation (isoflurane and natural sleep vs. active awake; Fig. 3*C*). We next investigated the effect of RSC memory ensemble stimulation on downstream areas that appear to be important for the rapid memory maturation effect described in this study; namely, the HPC and the ACC. We performed simultaneous local field potential (LFP) electrophysiological recordings from the RSC, ACC, and HPC while performing optogenetic stimulation of RSC memory ensembles during isoflurane, natural sleep, or active awake states in different groups (Fig. 4 and *SI Appendix*, Fig. S8). As expected, LFP traces during periods preceding RSC stimulation were more similar between the isoflurane and natural sleep groups, with a prominent delta band (1–4 Hz), in comparison with active awake, which had a prominent theta band (6–10 Hz) (Fig. 4*A* and *SI Appendix*, Fig. S8*B*). We subsequently performed high-frequency stimulation of memory ensembles in the RSC 1 d after CFC and compared the response to the stimulation in each of the three brain regions. To control for possible electrical artifacts arising from light stimulation, the response in each region was compared with the LFP power during an identical stimulation protocol performed before CFC, when mice were under a doxycycline-containing diet (Fig. 4*B*). The most evident response detected during RSC stimulation was an increase in the power in the stimulation frequency band (100 Hz) on the ACC and HPC electrodes, but to a lesser extent locally in the RSC, presumably because local recurrent circuits were less effectively activated than long-range projections (Fig. 4*C* and *SI Appendix*, Fig. S8 *F*–*H* for peri-stimulus time spectrograms and changes in other frequency bands). Notably, these responses were only observed during isoflurane and natural sleep, but not during the active awake state, consistent with the hypothesis that optogenetic stimulation of the RSC is capable of affecting network activity of these downstream areas only during specific brain states. Such propagation of long-range information may thus be essential for the apparent consolidation of memory described in this study.

Discussion

Our results demonstrate that high-frequency reactivation of RSC neural ensembles representing a recent CFC memory is sufficient to induce behavioral and neural circuit changes that resemble those observed during natural memory maturation after systems memory consolidation. Computational modeling suggests that systems consolidation is necessarily a slow process requiring multiple interleaved reactivation/retrieval events to avoid interference with previous memories and to allow integration of the new information with previously learned associations to produce a general schema (8, 30). This does not appear to be a requirement for the consolidation effect described here, and although we have not addressed the consequences of high-frequency stimulation on previously acquired information, our results suggest there is no natural physical or biochemical constraint to a more rapid consolidation of the memory being stimulated.

In this study, we used a high-frequency stimulation protocol that would allow induction of the type of neocortical synaptic changes that are thought to contribute to systems consolidation (20) without considering the timing and coordination of brain rhythms that are important for this process (sharp-wave ripples, thalamo-cortical spindles, cortical delta-waves) (31). This rhythmic activity is likely critical for inducing synaptic changes in selected memory ensembles during natural consolidation, but appears to be overcome by the strong and simultaneous activation of these ensembles with optogenetic stimulation. Such simultaneous reactivation is considerably different from natural spontaneous reactivations that preserve the temporal sequence of neural activity observed during learning (23, 32). The fact that concurrent activation of these ensembles in the RSC can lead to apparent systems consolidation or memory retrieval (26) suggests either that sequential

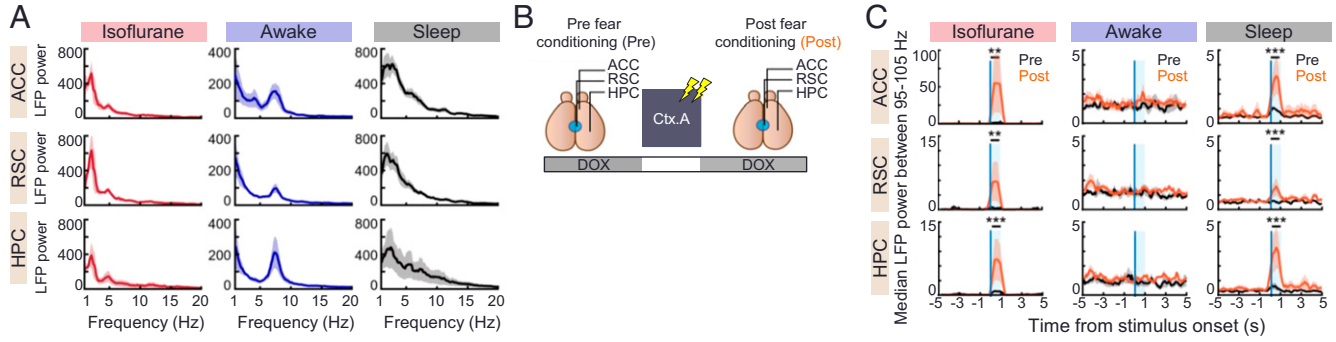

Fig. 4. Responses to RSC memory ensemble stimulation are differentially propagated depending on the brain state. (*A*) Average of power versus frequency plots for the first 40 s of recording during baseline from each brain region across all animals (n = 13). (*B*) Experimental protocol used for obtaining LFP recordings from the ACC, RSC, and HPC during control recordings performed before (Pre), and test recordings performed after CFC (Post). (*C*) The response to stimulation averaged across all mice for isoflurane (n = 5), active awake (n = 4), and natural sleep (n = 4). Curve plots represent mean ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$.

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neural firing is not important for these processes or that the mouse brain is able to rapidly adapt to the external stimulation and intrinsically generate neural sequences that are thought to be important for supporting these memory processes. This question could be addressed by performing multiunit recordings of neural ensembles during RSC stimulation, as recently demonstrated in the hippocampus using a similar transgenic system (33).

The RSC has been implicated in several cognitive tasks in humans, from spatial navigation (34), to prospective thinking (35) and autobiographical memory retrieval (36). In rodents, the RSC is necessary for retrieval of recent and remote contextual memories (37–39), spatial navigation (40), and sensory preconditioning (41), and it has been implicated in processing the conjunction between allocentric and egocentric spatial reference frames (42). Because of its extensive reciprocal connections with areas involved in recent and remote memory retrieval, such as the parahippocampus, hippocampus, anterior thalamus, and anterior cingulate cortex, the RSC has been proposed as an ideal candidate to relay hippocampal information broadly during systems consolidation (43). We have previously reported the existence of an RSC neural circuit capable of eliciting apparent memory retrieval on optogenetic stimulation 1 d after CFC (26). The finding that brief high-frequency stimulation of these same ensembles produces a memory with features of a consolidated remote memory suggests that systems consolidation is supported by activity-dependent plasticity produced by reactivation of neural ensembles involved in initial memory acquisition. This is consistent with replay models of systems consolidation in which circuit plasticity is thought to occur over a prolonged period through intermittent spontaneous reactivation of the learning-related neural ensembles (7, 8). It is interesting to note that reactivation of neural ensembles during awake states presented here does not appear to induce the emergence of the conditioned response (*SI Appendix*, Fig. S7), in contrast to what we have previously reported when using lower stimulation frequencies (26). This might explain why we did not detect any significant changes in natural memory retrieval after awake stimulation, as previously reported in mice (26) and humans (44) after induced memory reactivation. This result suggests that the type of activity underlying systems consolidation in memory-related ensembles might be different from the type of activity mediating normal memory retrieval.

Our results indicate that the ACC is an important neocortical area for the consolidation process described here. The ACC is a region of the medial prefrontal cortex that has reciprocal connections with the RSC, HPC, and is directly connected to the amygdala (45, 46). This area has been shown to be essential for retrieving remote CFC memories, but to be dispensable for retrieving recent memories in this task (9, 12, 47). A recent study examining the maturation of engram ensembles encoding a CFC memory in the medial prefrontal cortex (ACC and prelimbic cortex) found that immature ensembles are formed at the time of learning in this region and become functional only at remote points (48). This result is in line with previous studies demonstrating that neuronal plasticity occurs soon after learning even in brain areas that are not necessary to support the retrieval of a recent memory (49). Although we were not able to directly measure functional plasticity changes using the current methodology, we suggest that our optogenetic protocol accelerates the maturation of ACC ensembles by strengthening neocortical connections that allow the successful retrieval of the CFC memory in the absence of a functional HPC. Contrary to natural systems memory consolidation, the rapid stimulation-induced consolidation described here would occur at a time when the hippocampal component of the memory is usually engaged, and our immunohistochemistry data show that RSC optogenetic stimulation does not decrease HPC activity (Fig. 1*D* saline group). This result raises the possibility of the coexistence of two independent memory representations (hippocampal and cortical) capable of driving the retrieval process. This hypothesis is further supported by the observation that simultaneous, but not separate, inactivation of the ACC and HPC after optogenetic stimulation leads to memory impairment. Neocortical ensembles of CFC memories are thought to represent a schematic, less detailed representation of the original memory, capturing only general features of a given episode (50). The existence of a mature neocortical representation at a recent time could therefore induce memory generalization by being selectively recruited when only partial cues are presented at the time of retrieval (e.g., during context generalization), as previously suggested for CFC memories (51).

Finally, in our initial experiments, we used isoflurane to achieve a stable offline brain state that allowed us to complete the stimulation protocol without the animal waking up. This type of sedation is known to activate some of the same neural circuits involved in natural sleep (52–54), and to display similar neocortical activity to that observed during slow-wave sleep (55, 56), a brain state in which spontaneous replay of neural ensembles associated with recent experience has often been detected (23, 32) and that has been shown to be essential for memory consolidation in both humans and rodents (57, 58). The observation that rapid consolidation could be achieved during both sleep and light anesthesia, but not in active awake animals, indicates that the changes induced by the optogenetic protocol are dependent on brain state and rules out possible adverse effects of isoflurane for the observed effect. This result suggests that if natural systems consolidation follows a similar mechanism as the one described here, there might be some natural constraints on activity-induced systems consolidation, possibly to prevent reactivation activity from interfering with ongoing sensory processing or new memory encoding when animals are exploring the environment. In fact, it has been reported that artificial reactivation of memory ensembles in mice during active conscious states leads to false (59) and hybrid (60) memories. Some studies suggest that neural reactivations naturally occurring during wake and sleep periods might serve different purposes regarding the consolidation process, with reactivation events during sleep being less structured then reactivations observed during quiet awake periods (61). It is possible that the high-frequency reactivation of neural ensembles performed here generates different patterns of sequential activity depending on the brain state in which stimulation occurs, thus leading to different effects on systems consolidation. However, it should be noted that in the present study, we delivered optogenetic stimulation during active awake states, whereas spontaneous neural reactivations during awake states are mainly detected during quiet awake (62). Therefore, the physiological parameters and the contribution of quiet awake states for the apparent rapid consolidation described here remain to be addressed. Based on our electrophysiology recordings, it appears that the lack of induced consolidation when RSC ensembles are stimulated during active awake states might be explained by the way neural activity is propagated to other brain regions. In particular, our data demonstrate that high-frequency stimulation of RSC memory ensembles during active awake periods does not propagate to downstream areas, contrary to what is observed during stimulation in offline brain states (Fig. 4). Propagation of this activity during awake states could be prevented by gating by thalamocortical projections, direct regulation of local neocortical networks, or differences in the type of interneurons active during this brain state, as has been reported for state-dependent cortical processing of sensory information (63–66). Alternatively, propagation of activity could be identical for all brain states, but higher ongoing levels of network activity during awake conditions could interfere with or dilute the salience of the artificial reactivation.

Overall, our data are consistent with the view that the RSC is part of the hippocampal-cortical network that gradually modifies neocortical connections through reactivation of memory ensembles

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during natural systems consolidation and demonstrates that this process can be accelerated by direct stimulation of the relevant ensembles specifically during unconscious states.

Materials and Methods

In this study, we used male and female double-transgenic *cfos-tTA/tetO-ChEF* mice (26) bred against a C57BL/6NTac background. Single-transgenic littermates were used as wild-type controls and underwent the same experimental procedures as *cfos-tTA/tetO-ChEF*. All work was carried out under Institutional Animal Care and Use Committee-approved protocols in accordance with University of California, San Diego guidelines. Contextual fear conditioning and optogenetic reactivation of RSC memory ensembles were performed to test the role of ensemble reactivation on systems consolidation. For detailed methods and experimental procedures see in *SI Appendix, Methods*.

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