Generation of a Synthetic Memory Trace
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Generation of a Synthetic Memory Trace

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We investigated the effect of activating a competing, artificially generated, neural representation on encoding of contextual fear memory in mice. We used a *c-fos*–based transgenic approach to introduce the hM_3D_q DREADD receptor (designer receptor exclusively activated by designer drug) into neurons naturally activated by sensory experience. Neural activity could then be specifically and inducibly increased in the hM_3D_q-expressing neurons by an exogenous ligand. When an ensemble of neurons for one context (ctxA) was artificially activated during conditioning in a distinct second context (ctxB), mice formed a hybrid memory representation. Reactivation of the artificially stimulated network within the conditioning context was required for retrieval of the memory, and the memory was specific for the spatial pattern of neurons artificially activated during learning. Similar stimulation impaired recall when not part of the initial conditioning.

Direct electrical stimulation can be used to define functional domains in the brain, elicit stereotyped behavioral responses, drive self-stimulation behavior, and serve as conditioned or unconditioned stimuli in conditioning paradigms (*1*–*4*). This type of stimulation has typically been focal, using either microelectrodes or, more recently, genetically encoded mediators of neural excitability such as channelrhodopsin (*5*, *6*). Although such discrete, temporally coordinated, focal stimulation can drive behavior, we know much less about the effects of stimulating broadly distributed neural networks. The mammalian cortex displays substantial nonrandom, spontaneous neural activity that is internally generated rather than arising from sensory inputs, and this activity influences the processing of natural sensory stimuli (*7*–*10*). How does this internally generated activity influence the formation of a new memory representation?

To investigate this question, we used transgenic mice (Fig. 1A) in which the hM_3D_q receptor is expressed in an activity-dependent manner by a *c-fos* promoter–driven tTA transgene ($hM_3D_q^{fos}$ mice) (*11*, *12*). hM_3D_q is a G_q-coupled receptor that responds specifically to clozapine-*N*-oxide (CNO) and produces strong depolarization and spiking in pyramidal neurons (*12*). Transgenic mice exposed to a particular environmental stimulus will express hM_3D_q in those neurons that are sufficiently active to induce the *c-fos* promoter, and this naturally occurring neural ensemble can be subsequently reactivated artificially in the transgenic mice by delivery of CNO. Artificial activity induced in this manner will retain the spatial character of the neural ensemble, but will not preserve the temporal dynamics achieved by natural stimuli.

The expression of hM_3D_q is widely distributed in the brain of $hM_3D_q^{fos}$ double transgenic mice in the absence of doxycycline (Dox), enabling tTA-driven transcription (Fig. 1, B and C). Within a given brain area, expression is limited to a fraction of excitatory neurons that are sufficiently active to drive the *c-fos* promoter. Dox can be used to control the specific time window in which active neurons are genetically tagged with hM_3D_q by modulating tTA-driven transcription (*11*, *13*). To test the kinetics of CNO-based neural activation in these animals, we performed in vivo recording in the hippocampus of anesthetized animals. We found an increase in neuronal activity that reached a maximum intensity between 30 and 40 min after CNO injection (Fig. 1D). To examine the increase in neural activity more broadly, we used endogenous *c-fos* expression as an indicator of neural activity (Fig. 1, E and F). Relative to controls, we found significant increases (by a factor of 2 to 20) in *c-fos* labeling across multiple brain regions in CNO-injected $hM_3D_q^{fos}$ transgenic mice (table S1). Labeling for *c-fos* was found in both hM_3D_q-positive and hM_3D_q-negative neurons, with 91 ± 2% of hM_3D_q-positive neurons in CA1 colabeled with *c-fos* (fig. S2).

In standard contextual fear conditioning, animals develop a memory for the conditioning chamber in which they receive a footshock. The ability to form the context association is dependent on the hippocampus, which participates in encoding a representation of the environment (*14*, *15*). To test the effects of competing circuit activation on the formation of a memory trace, we designed the fear-conditioning protocol outlined in Fig. 2A. On day 1, $hM_3D_q^{fos}$ mice were exposed to a novel context (ctxA) in order to drive expression of the hM_3D_q transgene into neurons activated in that context. On day 2, mice were injected with Dox to inhibit further hM_3D_q receptor expression and with CNO to stimulate activity in the spatial pattern of neurons that expressed the receptor. The mice were then fear-conditioned in a distinct context (ctxB), and 24 hours later, memory performance was tested in the absence and presence of CNO. Thus, we fired the neurons active in ctxA while the mice were fear-conditioned in ctxB.

We anticipated three potential outcomes. The strong synthetic activation of ctxA neurons could be dominant and serve as a conditioned stimulus

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to produce an associative fear memory. This would lead to a fear response to CNO, or possibly even a fear response to ctxA itself, if the artificial and natural activation of the neurons were sufficiently similar. This was not observed, as the level of freezing in ctxA was not significant in transgenic mice either with or without CNO injection (Fig. 2B). A protocol in which ctxA neurons were activated by CNO and mice were shocked immediately in ctxB [to prevent formation of a ctxB representation (*13*)] also failed to produce a CNO-dependent memory (fig. S3). Similarly, when the neurons active during conditioning itself were tagged with the hM_3D_q transgene, CNO did not produce significant freezing (fig. S5). Thus, the synthetic activity alone could not serve as a conditioned stimulus in fear conditioning. A second possibility was that the natural sensory experience in ctxB would dominate and transgenic mice would show normal conditioning to ctxB. The $hM_3D_q^{fos}$ mice displayed a severe deficit in freezing to ctxB, which suggests that the CNO-induced activity was interfering with normal encoding of memory for ctxB (Fig. 2C). A third possibility was that mice would form a hybrid representation, incorporating elements of both the CNO-induced artificial stimulation and the natural sensory cues from ctxB. This appears to be the case, as the transgenic mice showed a significant increase in freezing in response to CNO delivered in the ctxB setting during the 24-hour memory test (Fig. 2C).

We observed similar results in two separate experiments when a different contextual setup for ctxA neural labeling was used (figs. S1 and S4). The requirement for reactivation of the transgene-expressing neurons during memory retrieval suggests that their activity was incorporated into the memory trace. Consistent with this idea, we found a correlation between freezing during memory retrieval and the degree of neural activation, as assessed by *c-fos* expression in the hippocampus (Fig. 2, D and E).

Retrieval of a memory representation likely involves the reactivation of some neurons that were active during the initial learning (*11*, *16*–*18*). To test the susceptibility of this spatial code to competing neural network activation, we exposed $hM_3D_q^{fos}$ mice to ctxA to allow expression of the hM_3D_q transgene but then conditioned them in ctxB without CNO stimulation of the ctxA neural ensemble (Fig. 3). As expected, these mice developed wild-type levels of freezing to ctxB 24 hours after conditioning. Now, however, activation of the hM_3D_q-expressing neurons impaired memory performance during retrieval in ctxB. This suggests that CNO-induced activation of a competing neural network interferes with the learned spatial code and degrades recognition if this activity was not present during the initial training. This is not surprising, given that even limited focal hippocampal stimulation has been shown to disrupt spatial memory (*19*).

Does the hybrid fear memory formed by $hM_3D_q^{fos}$ mice incorporate the specific pattern of ctxA neurons activated by CNO during learning, or are the mice responding to a less specific alteration in brain state? To distinguish between these possibilities, we conditioned mice in the presence of CNO-induced firing of ctxA-labeled neurons but then placed the mice on Dox to allow turnover of the hM_3D_q receptor. Two days later, we removed Dox from the animals' diet and placed them in a new home cage to allow de novo expression of the hM_3D_q receptor in a distinct group of neurons (ctxC). Fourteen days after initial conditioning, we tested memory performance as assessed by freezing scores in ctxB in the absence and presence of CNO-induced synthetic activation. We found no increase in freezing in $hM3D_q^{fos}$ mice in response to CNO (Fig. 4, A

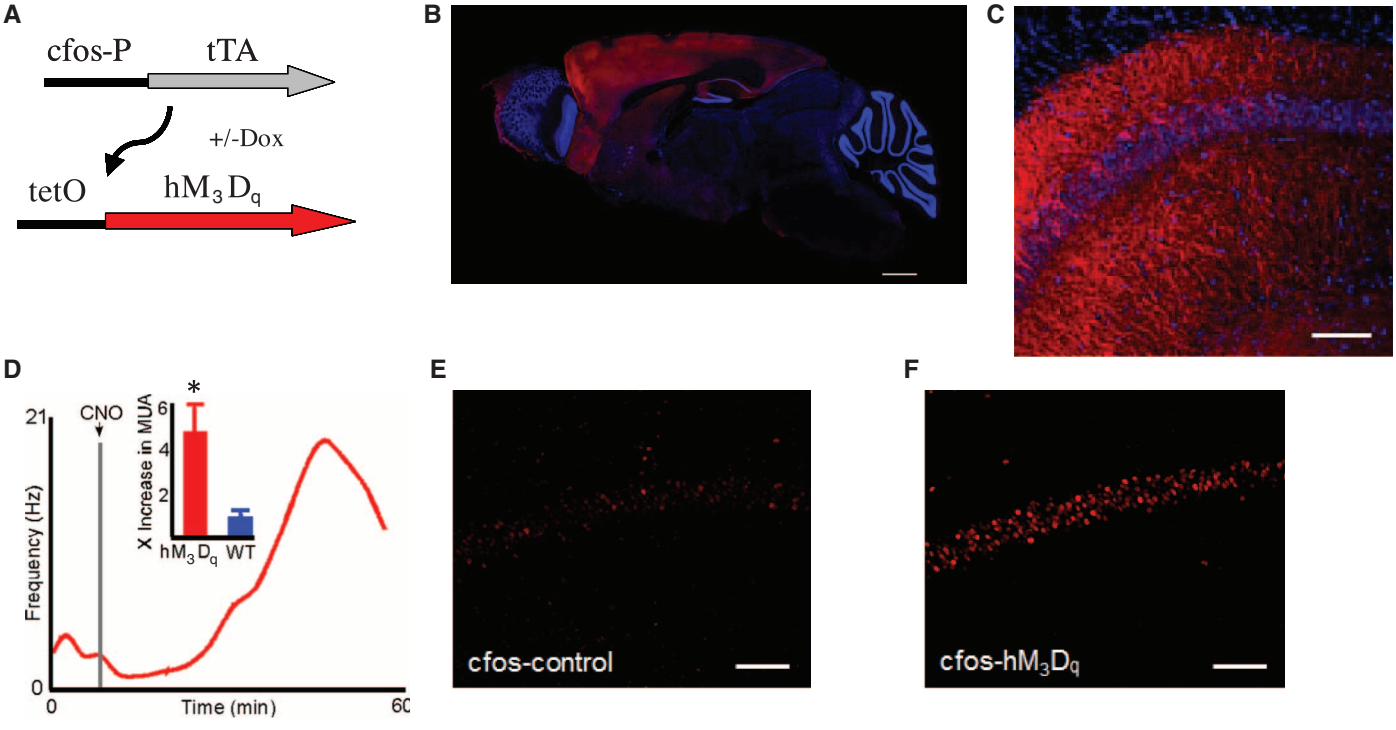

Fig. 1. Expression and activation of the hM_3D_q transgene. (**A**) Transgenic mice used in this study carry two transgenes. The first expresses the tetracycline transcriptional activator (tTA) under control of the activity-regulated *c-fos* promoter. The second transgene allows expression of hM_3D_q under the tet operator (tetO), which is activated upon binding of tTA but is inhibited by Dox. (**B**) Overall spatial expression profile of the hM_3D_q transgene in mice off Dox maintained in the home cage. Immunofluorescence was strong in hippocampus, in basolateral amygdala, and throughout the cortex. Fluorescence was also observed to a small extent in the pontine nucleus and brainstem. Scale bar, 1000 μm. (**C**) Expression in the CA1 region of the hippocampus, showing sparse and distributed expression of the hM_3D_q transgene. Scale bar, 100 μm. (**D**) CNO injection caused increased neural activity in $hM_3D_q^{fos}$ mice. Red curve shows multi-unit activity (MUA) recorded from dorsal CA1 of an anesthetized $hM_3D_q^{fos}$ mouse over time. Inset shows relative increase in MUA [mean MUA 30 to 40 min after injection compared to mean pre-injection baseline, 4.76 for $hM_3D_q^{fos}$ mice ($n = 6$) versus 0.9 for wild-type (WT) mice ($n = 6$); Wilcoxon signed-rank test, $^*P < 0.01$]. (**E** and **F**) *c-fos* induction 1.5 hours after CNO administration in a control (left) and $hM_3D_q^{fos}$ (right) mouse. $hM_3D_q^{fos}$ mice showed on average a factor of 2.5 increase in *c-fos* expression in the hippocampal CA1 region relative to control mice (see table S1; $hM_3D_q^{fos}$, $n = 10$; control, $n = 10$; t test, $P < 0.02$). Scale bars, 100 μm.

and C); this finding demonstrates a requirement for reactivation specifically of the learned (ctxA) neural ensemble, rather than a generalized change in brain state caused by CNO-induced activity.

To further address the issue of ensemble specificity, we preexposed mice to the fear-conditioning context (ctxB) on day 1 to express the hM_3D_q receptor in neurons that were activated in that context. We reasoned that the synthetic activation of this pattern of neurons would more likely overlap with the natural activity during learning in ctxB and should therefore not interfere with the production of a normal ctxB representation. When mice were fear-conditioned after injection of CNO to artificially activate the ctxB ensemble during learning, they developed wild-type levels of 24-hour context fear memory that were independent of CNO stimulation (Fig. 4, B and D). This is in contrast to the deficit produced in mice preexposed to the novel ctxA and further supports the hypothesis that there must be a match in the spatial pattern of neural activity at learning and retrieval.

Several recent studies have suggested flexibility in the specific neurons incorporated into a fear memory trace in the amygdala through a selection mechanism in which more excitable neurons are preferentially incorporated into the trace (*16*–*18*). Our results do not appear to be attributable to this type of selection, as the reactivation of the neurons with CNO is required for retrieval, whereas in the previous studies the stimulated neurons were part of a representation that could be naturally retrieved. This difference may be due to different requirements for forming simple associations in the amygdala versus more complex representations in the hippocampus and cortex.

In our study, the artificially stimulated neural ensembles become incorporated into the memory and the amount of activation in CA1 and CA3 during retrieval is correlated with the strength of memory performance in transgenic mice. In one recent study, ChR2 stimulation of a random population of neurons in the piriform cortex combined with odorant during conditioning found that either the artificial stimulation or the odorant alone could produce recall, which suggests independent and noninterfering representations (*20*). In contrast, we found that the CNO activation alone could not act as an independent cue. These studies differed in a variety of parameters, including anatomy and size of the artificially stimulated ensembles; one critical difference may be that the activity induced by hM_3D_q is not temporally coordinated in response to the inducing stimulus (CNO), as is the case with ChR2-driven stimulation by light. However, the sensory input during conditioning and retrieval in ctxB may coordinate the activity of the CNO-depolarized cells to provide some degree of temporal coordination to the CNO-driven neurons and account for the requirement for the compound stimulus. Alternatively, it is possible that the uncoordinated

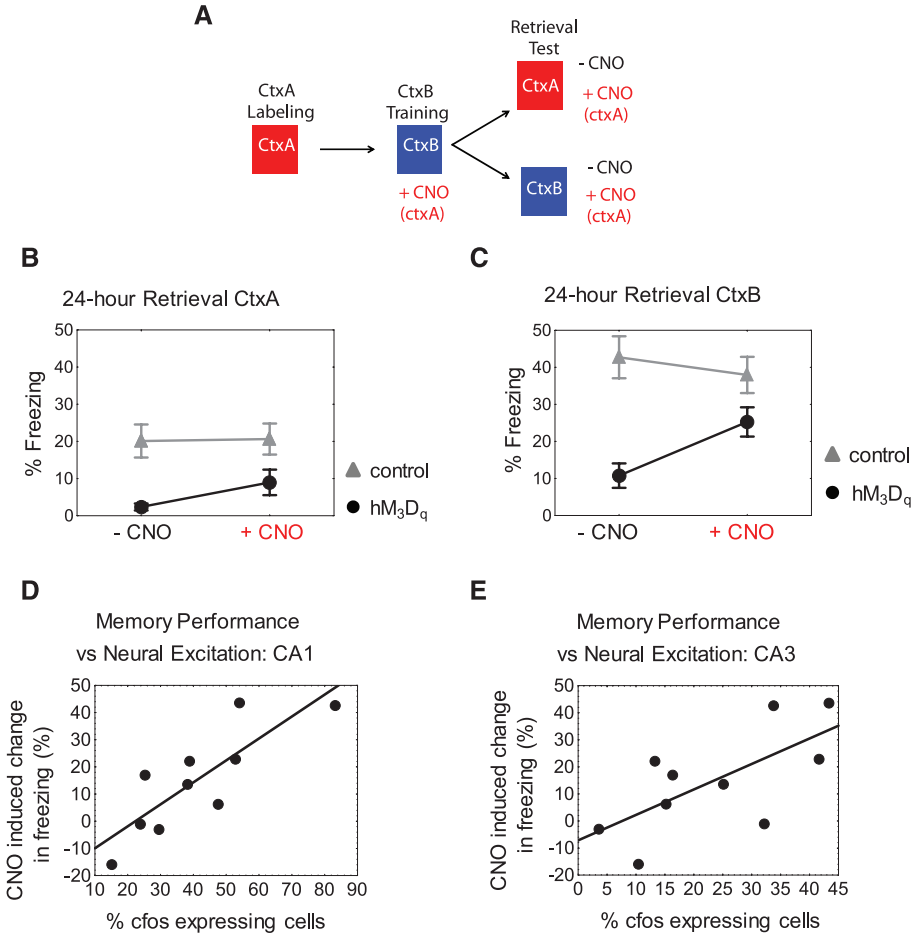

Fig. 2. Incorporation of synthetic neural activity into a 24-hour memory representation. (**A**) Schematic of experimental procedure. (**B**) Freezing in ctxA 24 hours after conditioning in ctxB. $hM_3D_q^{fos}$ mice ($n = 14$) froze significantly less than did control mice ($n = 13$) in ctxA in both the absence and presence of CNO [repeated-measures analysis of variance (ANOVA): main effect of genotype, $F(1,26) = 10.96$, $P < 0.005$]. CNO had no significant effect on freezing in either group (post hoc Bonferroni test: $hM_3D_q^{fos}$, $P = 0.192$; control, $P = 1.00$). (**C**) Transgenic $hM_3D_q^{fos}$ mice showed impaired 24-hour memory for ctxB that was rescued by injection of CNO [repeated-measures ANOVA: genotype × CNO interaction, $F(1.25) = 10.15$, $P < 0.005$]. $hM_3D_q^{fos}$ mice froze significantly less than did control mice in ctxB in the absence of CNO (post hoc Fisher's least significant difference test; $P < 0.001$) but were statistically similar in ctxB in the presence of CNO ($P = 0.117$) and showed a significant increase in freezing in ctxB with CNO relative to ctxB alone ($P < 0.001$). (**D** and **E**) Correlation between the difference in freezing scores in the presence and absence of CNO and endogenous *c-fos* expression 1 hour after memory testing in hippocampal areas CA1 (D) ($r = 0.8276$, $P < 0.005$) and CA3 (E) ($r = 0.6742$, $P < 0.05$).

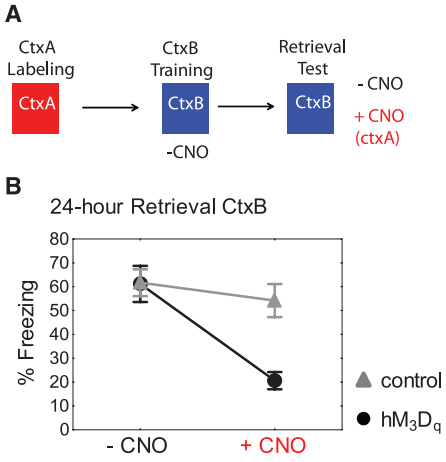

Fig. 3. Disruption of memory retrieval by synthetic neural activation. (**A**) Schematic of experiment. (**B**) Transgenic $hM_3D_q^{fos}$ mice developed a normal 24-hour context memory when conditioned in the absence of CNO. This memory was disrupted by CNO injection to activate the competing ctxA representation [$hM_3D_q^{fos}$, $n = 12$; control, $n = 12$; repeated-measures ANOVA: main effect of genotype, $F(1,22) = 5.3$, $P < 0.05$; CNO, $F(1,22) = 28.6$, $P < 0.001$; genotype × CNO interaction, $F(1,22) = 13.5$, $P = 0.001$]. hM_3D^{fos} mice froze significantly less in the presence of CNO relative to before CNO administration (post hoc Fisher's least significant difference test; $P < 0.001$) and froze significantly less than did control mice in the presence of CNO ($P < 0.001$).

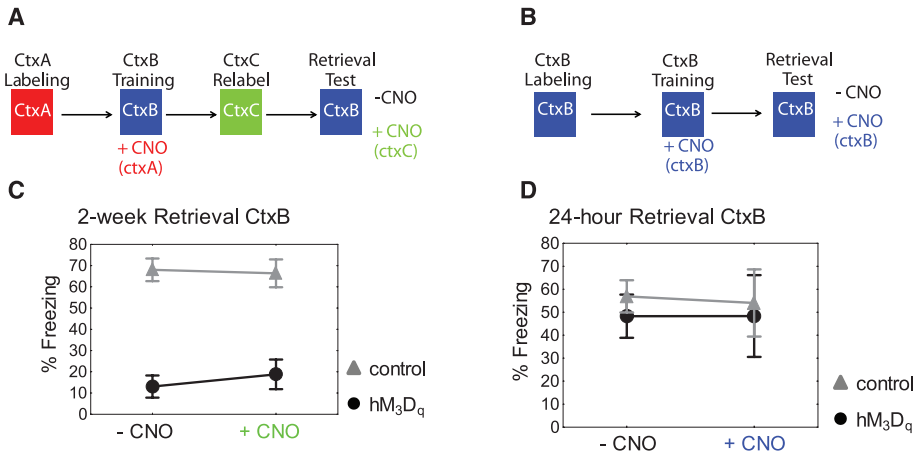

Fig. 4. Memory performance during synthetic reactivation is network-specific. (**A** and **C**) When CNO-induced synthetic activation did not occur in identical neural populations during memory formation and memory retrieval, a memory deficit was observed. $hM_3D_q^{fos}$ mice showed significantly less freezing than did control mice in ctxB, both in the absence and presence of CNO [$hM_3D_q^{fos}$, $n = 14$, control, $n = 17$; repeated-measures ANOVA: main effect of genotype, $F(1,23) = 51.15$, $P < 0.001$]. (**B** and **D**) When $hM_3D_q^{fos}$ mice were exposed to ctxB off of Dox to induce hM_3D_q expression and then fear-conditioned on Dox after CNO injection in ctxB, synthetic activation by CNO was not necessary for memory recall in ctxB [ctxB – CNO: $hM_3D_q^{fos}$, $n = 9$; control, $n = 10$; ctxB + CNO: $hM_3D_q^{fos}$, $n = 5$; control, $n = 6$; repeated-measures ANOVA: $F(2,18) = 0.0474$, $P = 0.954$].

CNO-based stimulus could serve as a conditioned stimulus if it was limited to a discrete primary sensory area, such as the piriform cortex.

Current views of sensory processing recognize the role of internally generated (spontaneous) neural activity in generating a representation from a given sensory input (*8*). This activity is not random but has spatial and temporal structure that is thought to represent defined ensembles formed through previous learning-related plasticity. Moreover, in psychology, the idea of a schema as a preexisting framework of relationships that modulates learning suggests that new memories are not produced de novo; rather, coding of new learned information depends on preexisting circuit activity (*21*, *22*). Although the CNO-based stimulation does not replicate the temporal dynamics of this naturally occurring internal activity, the approach allows the activation of a distributed spatial pattern of neurons recruited during a specific experience (ctxA exposure). Our results show that this spatial pattern of activity at the time of learning and retrieval must match for appropriate recall. The results imply a strong spatial component to coding in this form of learning and support the idea that the internal dynamics of the brain at the time of learning contribute to memory encoding.

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Supporting Online Material

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