Covert capture and attenuation of a hippocampus-dependent fear memory

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Reconsolidation may be a viable therapeutic target to inhibit pathological fear memories. In the clinic, incidental or imaginal reminders are used for safe retrieval of traumatic memories of experiences that occurred elsewhere. However, it is unknown whether indirectly retrieved traumatic memories are sensitive to disruption. Here we used a backward (BW) conditioning procedure to indirectly retrieve and manipulate a hippocampus (HPC)-dependent contextual fear engram in male rats. We show that conditioned freezing to a BW conditioned stimulus (CS) is mediated by fear to the conditioning context, activates HPC ensembles that can be covertly captured and chemogenetically activated to drive fear, and is impaired by post-retrieval protein synthesis inhibition. These results reveal that indirectly retrieved contextual fear memories reactivate HPC ensembles and undergo protein synthesis-dependent reconsolidation. Clinical interventions that rely on indirect retrieval of traumatic memories, such as imaginal exposure, may open a window for editing or erasure of neural representations that drive pathological fear.

Cognitive behavioral therapies, such as prolonged exposure therapy, are treatments widely used for a number of debilitating fear-related and anxiety disorders1,2. Similar to extinction learning in rodents, prolonged exposure therapy attempts to extinguish maladaptive fear responses by exposing patients to trauma-related stimuli (often using imaginal exposure) in a safe environment. Despite efficacy in most patients, clinical interventions are nonetheless susceptible to relapse3. Accordingly, there is substantial interest in developing therapeutic strategies that selectively target and eliminate traumatic fear memories.

Studies in rodents have shown that consolidated fear memories become labile following retrieval and undergo a protein-synthesis-dependent phase of reconsolidation4-6. Memory attenuated in this way may be less susceptible to relapse7, suggesting an effective therapeutic strategy to provide long-term relief4. Although reconsolidation-based therapies have high therapeutic potential8, translation of findings from experimentally controlled situations to real-world clinical scenarios is a challenge. In animal models, for example, contextual fear memories are reactivated by direct exposure to shock-associated contexts9,10. In patients, however, these memories must be retrieved indirectly using trauma-related cues or imaginal exposure. Although the development of virtual reality exposure therapy holds promise for enhancement of exposure-based treatment outcomes in humans11, a critical question is whether reactivation using indirect reminders yields episodic retrieval of traumatic fear memories that are sensitive to reconsolidation manipulations12,13.

To accomplish ‘covert’ memory retrieval in rats, we utilized a BW fear-conditioning procedure14,15. Critically, this procedure does not require returning the animal to the conditioning context to retrieve an averse memory of that place. In this procedure, rats are placed into a novel chamber and presented with several trials in which an aversive footshock unconditioned stimulus (US) is immediately followed by the presentation of an auditory CS. In this procedure, the CS does not become directly associated with the US but nevertheless evokes conditioned fear (indexed by freezing behavior). It does so by reactivating a memory of the conditioning context and indirectly retrieving a memory of the aversive US16. Given the critical role for the HPC in contextual fear memory17, we hypothesized that a BW CS reactivates a contextual fear engram in the HPC in the absence of re-exposure to the conditioning context. This would allow for the capture and manipulation of an indirectly retrieved contextual fear memory, similar to the way in which a clinician might use an incidental reminder to facilitate the episodic recollection of a traumatic experience in the clinic.

Results

Effects of context extinction on fear to a FW or BW CS. To demonstrate that conditioned freezing to a BW CS is mediated by fear to the conditioning context, animals underwent forward (FW) or BW conditioning followed by extinction of the conditioning context (Fig. 1a). We hypothesized that context extinction would undermine freezing to the BW but not the FW CS. During conditioning (Fig. 1b), all rats exhibited low freezing before the first trial but showed increased freezing across the conditioning trials (repeated measures: main effect of trial, F(4, 112) = 99.7, P < 0.0001). On the following 2 days, half of the rats in each group were placed into the conditioning context (A, ’Ext’), while the other half were simply exposed to a novel context (C, ’NoExt’) for an equivalent amount of time. As expected, freezing behavior in rats exposed to the conditioning context was elevated initially and decreased across two days; rats exposed to the neutral context showed low levels of freezing behavior in both sessions. A repeated-measures analysis of variance (ANOVA) revealed a main effect of time (F(1, 28) = 14.4, P = 0.0007), a main effect of extinction procedure (F(1, 28) = 10.2, P = 0.003) and a significant time × extinction interaction (F(1, 28) = 14.6, P = 0.0007). Importantly, there were no statistical differences between groups in regard to average freezing during the second day of extinction (P > 0.11; Fig. 1b).

Twenty-four hours after the final extinction session, all rats were tested for conditioned freezing to the FW or BW CS (Fig. 1b). Analysis of freezing across the five test trials (excluding baseline (BL)) revealed a main effect of trial (repeated measures, F(4, 112) = 8.04, P < 0.0001), a main effect of conditioning procedure...
Effects of CS exposure on c-Fos activity in the dHPC. Given that freezing to BW CS is mediated by retrieval of a contextual fear memory, we asked whether the BW CS engages the dorsal hippocampus (dHPC), a brain region known to be important for both contextual fear and higher-order conditioning. Three experimental groups were compared: rats conditioned and tested to a FW CS, rats conditioned and tested to a BW CS and rats conditioned to either a FW or BW CS (evenly split) but remaining in their home cage during the retrieval session (‘NoTest’). Before conditioning, rats underwent a habituation session in what would later be the test context (Fig. 1c shows the behavioral schematic). This session was conducted in an effort to bias c-Fos expression towards cells activated by CS retrieval rather than the test context. Twenty-four hours after habituation, rats underwent FW or BW conditioning in a distinct context. Freezing was low during the BL period and increased significantly across the duration of the session (main effect of trial, $F(4, 76) = 143.3$, $P < 0.0001$; Fig. 1d). Although the analysis revealed a significant trial $\times$ conditioning procedure interaction ($F(4, 76) = 2.54$, $P = 0.047$), post hoc comparisons indicated that there were no statistical differences between any of the groups across the conditioning session ($P > 0.47$). Twenty-four hours after conditioning, rats received a retrieval test in a familiar, safe context; control rats (NoTest) remained in their home cage and were perfused alongside retrieval animals (Fig. 1d). During the retrieval test, freezing was low before the first trial and was significantly increased by CS presentation in both FW and BW conditioned rats (main effect of trial: repeated measures, $F(1, 21) = 18.6$, $P = 0.0003$; no other main effects or interactions, $F < 2.98$, $P > 0.09$).

Ninety minutes after the retrieval test, rats were killed and their brains processed for c-Fos immunohistochemistry; c-Fos-positive (c-Fos+) nuclei were counted in three dHPC subregions (Fig. 1e). As shown in Fig. 1f, presentation of either the FW or BW CS increased the number of c-Fos+ cells in the dHPC relative to NoTest controls. One-way ANOVA comparing c-Fos counts within each region revealed significant main effects of group in dorsal CA1 (dCA1) ($F(2, 20) = 12.90$, $P = 0.0003$), dorsal dentate gyrus (dDG) ($F(2, 20) = 3.61$, $P = 0.04$) and a trend in dorsal CA3 (dCA3) ($F(2, 20) = 3.47$, $P = 0.051$). Within dCA1, both FW and BW CS produced similar increases in the number of c-Fos+ cells relative to NoTest controls (BW versus NoTest, $P < 0.0001$; FW versus NoTest, $P = 0.004$), whereas within the DG the BW CS produced greater increases in the number of c-Fos+ cells relative to all other groups (BW versus NoTest, $P = 0.027$; BW versus FW, $P = 0.037$; Fig. 1f). These findings reveal that the dHPC is engaged during expression of conditioned freezing, and that the DG may be preferentially engaged by the contextual memory retrieved by a BW CS.

Impact of CS exposure on c-Fos activity in a dHPC fear engram. An important question is whether presentation of the BW CS during a retrieval test reactivates DG cells active during BW conditioning. To examine this possibility, we infused the dHPC with a viral cocktail (adeno-associated viruses for Fos-dependent tetracycline-controlled transactivator protein (AAV-Fos-tTA) and tetracycline response element-regulated hM3Dq-mCherry protein (AAV-TRE-hM3Dq-mCherry)) to achieve activity-dependent expression of ‘designer receptors exclusively activated by designer drugs’ (DREADDs; Fig. 2a,b). To restrict tTA-dependent expression of hM3Dq-mCherry to the conditioning session, rats were maintained on a doxycycline (DOX) diet until conditioning.

Before conditioning, rats were given a brief exposure session in which they were habituated to the retrieval context and were immediately taken off DOX (OfDox) to open a cell-labeling window for the conditioning session (Fig. 2c). Two days later, all rats underwent BW conditioning and were immediately placed back on DOX (OnDox). Conditioning was similar to previous experiments (main effect of trial: $F(4, 40) = 71.5$, $P < 0.0001$); there were no other main effects or interactions ($F < 0.29$, $P > 0.74$; Fig. 2d). The next day, half of the rats were given a BW CS (Ret) retrieval session to examine the extent to which cells activated within the DG during conditioning (mCherry) were reactivated by the presentation of the BW CS (overlapping endogenous c-Fos protein); the other half of the rats served as controls and were simply exposed to the retrieval context for an equivalent amount of time. Note that animals did not receive drug injections for this test; hM3Dq-mCherry labeling was simply used as a proxy for dHPC activity at conditioning.

Analysis of freezing across the five-trial retrieval test (Fig. 2d) revealed no differences between groups (no main effect of group or trial $\times$ group interaction, $F < 1.89$, $P > 0.11$). However, close inspection of the data revealed that average freezing across the first two trials was significantly elevated in rats that were presented with the CS (main effect of group: repeated measures $F(1, 10) = 4.97$, $P = 0.049$). Importantly, although we found no differences between groups in the overall number of cells activated by conditioning (hM3Dq-mCherry+) or CS retrieval (c-Fos+), rats that received BW CS presentations during the retrieval test displayed a significant increase in the percentage of cells that were double labeled (Fig. 2b,c,e; unpaired Student’s two-tailed t-test, $t(10) = 3.09$, $P = 0.012$).

Fig. 1 | Conditioned freezing to a BW CS is mediated by a contextual fear memory and engages the dHPC. a, Behavioral schematic. b, Freezing behavior during conditioning, extinction and retrieval testing. For conditioning, the far left panel depicts mean percentage freezing for each group during the 5-min BL and across each conditioning block. For extinction, data are shown as the mean percentage freezing across the entire session for each day. For CS retrieval, data represent mean percentage freezing during the 5-min BL and across each test trial (each trial was composed of a 10-s CS and a 60-s ISI). The panel at far right depicts average freezing across all test trials. Although extinction of the conditioning context did not significantly affect freezing to the FW CS, it significantly reduced freezing elicited by the BW CS (BW-NoExt versus BW-Ext, $P = 0.005$), two-way repeated-measures ANOVA followed by Bonferroni’s multiple comparisons post hoc test. Groups: FW-NoExt, $n = 8$; FW-Ext, $n = 8$; BW-NoExt, $n = 8$; BW-Ext, $n = 8$. c, Behavioral schematic. d, Freezing behavior during conditioning and retrieval. For conditioning, the left panel depicts freezing during the 5-min BL period and across each conditioning block. For retrieval, the right panel depicts average freezing across four retrieval trials (each trial composed of a 10-s CS and a 60-s ISI). Animals were killed for c-Fos immunohistochemistry 90 min after the first retrieval trial. e, Representative photomicrograph depicting c-Fos labeling and regions (dDG, dCA3, and dCA1) counted within the dHPC (m, medial; d, dorsal). f, Mean c-Fos+ cells for each of the quantified regions (four to six images per mouse, standardized to 0.1 mm$^2$). In CA1, presentation of either the BW or FW CS resulted in elevated levels of c-Fos expression relative to controls (BW versus NoTest, $P < 0.0001$; FW versus NoTest, $P = 0.004$, whereas in DG the BW CS resulted in increased levels of c-Fos relative to all other groups (BW versus NoTest, $P = 0.027$; BW versus FW, $P = 0.037$), one-way ANOVA (per region) followed by Fisher’s PLSD post hoc test. *$P < 0.05$, **$P < 0.005$. Groups: FW, $n = 7$; BW, $n = 8$; NoTest, $n = 8$. All data are represented as means $\pm$ s.e.m.
This suggests that presentation of the BW CS resulted in reactivation of neural ensembles within the DG that encode contextual representations during BW conditioning.

**Chemogenetic activation of a covertly captured HPC ensemble.** Collectively, these experiments suggest that the BW CS functions as an indirect retrieval cue to covertly reactivate a HPC-dependent contextual fear memory. If so, chemogenetic activation of a covertly captured HPC ensemble should be sufficient to drive conditional fear in a safe context, as has been demonstrated for direct reactivation of HPC ensembles\(^\text{18}\). Accordingly, rats were injected with the same viral cocktail described above to achieve DOX-regulated and c-Fos-dependent expression of the chemogenetic actuator hM3Dq-mCherry in the dHPC. Before conditioning, and while on the DOX diet, all rats were habituated to the retrieval context in an effort to minimize their tendency to generalize fear across contexts (Fig. 3a). The next day, all rats underwent BW conditioning. All groups exhibited reliable conditioning (main effect of trial: repeated

```-conditioning
FW-NoExt 4 3 2 1
FW-Ext 0 1 2 3
BW-NoExt 0 1 2 3
BW-Ext 0 1 2 3
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**CS retrieval**

```
FW-NoExt 4 3 2 1
FW-Ext 0 1 2 3
BW-NoExt 0 1 2 3
BW-Ext 0 1 2 3
```

**CS retrieval (average)**

```
FW-NoExt 4 3 2 1
FW-Ext 0 1 2 3
BW-NoExt 0 1 2 3
BW-Ext 0 1 2 3
```

**Context extinction**

```
FW-NoExt 4 3 2 1
FW-Ext 0 1 2 3
BW-NoExt 0 1 2 3
BW-Ext 0 1 2 3
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**Context extinction (or exposure)**

```
FW-NoExt 4 3 2 1
FW-Ext 0 1 2 3
BW-NoExt 0 1 2 3
BW-Ext 0 1 2 3
```

**Context extinction**

```
FW-NoExt 4 3 2 1
FW-Ext 0 1 2 3
BW-NoExt 0 1 2 3
BW-Ext 0 1 2 3
```

**Context extinction (or exposure)**

```
FW-NoExt 4 3 2 1
FW-Ext 0 1 2 3
BW-NoExt 0 1 2 3
BW-Ext 0 1 2 3
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**Context extinction (or exposure)**

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FW-NoExt 4 3 2 1
FW-Ext 0 1 2 3
BW-NoExt 0 1 2 3
BW-Ext 0 1 2 3
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**Context extinction (or exposure)**

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FW-NoExt 4 3 2 1
FW-Ext 0 1 2 3
BW-NoExt 0 1 2 3
BW-Ext 0 1 2 3
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Two days later, rats were given a retrieval session in which they were presented with the BW CS to capture and tag active HPC ensembles; after the retrieval session they were immediately placed back on DOX. Analysis of freezing behavior across the five-trial retrieval session revealed a significant main effect of trial (repeated measures: $F(4, 144) = 145.3, P < 0.0001$). There were no other significant main effects or interactions ($F < 1.8, P > 0.15$). After conditioning, rats were immediately returned to their home cages and the DOX diet was replaced with normal chow.

Two days later, rats were given a retrieval session in which they were presented with the BW CS to capture and tag active HPC ensembles; after the retrieval session they were immediately placed back on DOX. Analysis of freezing behavior across the five-trial retrieval session revealed a significant main effect of trial (repeated measures: $F(4, 144) = 145.3, P < 0.0001$), a significant main effect of group ($F(3, 36) = 4.00, P = 0.015$) and a significant trial x group interaction ($F(15, 180) = 3.41, P < 0.0001$). Similar to our previous experiment, we found that freezing was maximal during the first two retrieval trials and was significantly elevated in rats presented with a CS (Fig. 3b; main effect of Ret versus NoRet; repeated measures: $F(1, 38) = 11.7, P = 0.002$). Importantly, presentation of the BW CS increased hM3Dq-mCherry expression in animals removed from the DOX diet relative to control rats that remained on DOX throughout the duration of the experiment (main effect of group: factorial ANOVA, $F(3, 16) = 41.55, P < 0.0001$). Post hoc analyses confirmed that rats remaining on DOX were statistically different to all other groups ($P < 0.0001$; Fig. 3c,d).

Twenty-four hours after the retrieval session, rats received systemic injections of either vehicle (Veh) or the DREADD ligand, clozapine-N-oxide (CNO, 3 mg kg^{-1}), to activate the captured HPC ensemble; freezing responses were assessed during a 10-min test session in a novel context. As shown in Fig. 3b, CNO increased freezing behavior in rats that received the BW CS when off DOX (Ret-CNO) relative to all other control groups. Repeated-measures ANOVA revealed a main effect of group ($F(3, 36) = 7.94, P = 0.0003$); there were no other significant main effects or interactions ($F < 1.6, P > 0.14$). Post hoc comparisons confirmed that freezing behavior in the Ret-CNO group was significantly elevated relative to controls ($P < 0.005$). This indicates that chemogenetic
**Chemogenetic activation of a covertly captured HPC neural ensemble drives freezing behavior.**

For conditioning (covert capture), the panel depicts average freezing during the 3-min BL and across conditioning blocks. For retrieval (covert capture), the panel depicts average freezing during the 3-min BL and average freezing across the first two retrieval trials (each trial consists of a 10-s CS and a 60-s ISI). During the covert capture session, animals were removed from the DOX diet to capture dHPC ensembles activated by presentation of the BW CS. During the test session (engram activation), systemic CNO administration increased freezing in Ret-CNO relative to all other groups. The far right panel shows average freezing across the engram activation session for each group (Ret-CNO versus Ret-Veh, P = 0.004; Ret-CNO versus NoRet-CNO, P < 0.0001; Ret-CNO versus Ret-CNO-OnDOX, P < 0.0001). Groups: NoRet-CNO, n = 8; Ret-CNO, n = 11; Ret-CNO-OnDOX, n = 8; NoRet-CNO, n = 9; Ret-Veh, n = 7; Ret-CNO, n = 9; Ret-Veh, n = 5; Ret-CNO, n = 5. All data are represented as means ± s.e.m. **P < 0.005, ***P < 0.0001. i.p., intraperitoneal.

**Reactivation of the HPC neuronal ensemble representing the contextual memory and covertly retrieved by a BW CS is sufficient to drive conditional freezing.**

As shown in Fig. 3d, mCherry labeling was increased in all animals undergoing a retrieval test when off the DOX diet, independent of whether the BW CS was presented (Ret-CNO; Ret-Veh) or not (NoRet-CNO). This indicates that context exposure alone was sufficient to drive activity-dependent expression of hM3Dq in the dHPC, and implies that this may have accounted for mCherry expression in those animals also presented with the BW CS. Moreover, CNO delivery increased c-Fos expression in both the NoRet-CNO and Ret-CNO groups, as well as the total number of...
cells positive for both c-Fos and hM3Dq-mCherry (co-labeled) within the DG. Critically, however, only rats in the Ret-CNO group exhibited increased levels of freezing behavior after CNO administration. This suggests that cells tagged after presentation of the BW CS (Ret-CNO), but not mere placement in the retrieval context (NoRet-CNO), represent a contextual fear memory.

Inhibition of protein synthesis in the dHPC following retrieval of a FW or BW CS. These experiments support the hypothesis that a BW CS evokes freezing behavior by retrieving a HPC-dependent contextual fear engram. This suggests that the BW CS serves as an indirect retrieval cue to covertly access a contextual fear memory in the HPC. Although directly reactivated contextual fear memories undergo a period of reconsolidation in which they are sensitive to protein synthesis inhibition, it is not known whether this is true for clinically relevant indirect retrieval procedures. To explore this question, rats were implanted with bilateral cannulae targeting the dorsal DG and, after recovery, were subject to either FW or BW fear conditioning (Fig. 4a–c). During conditioning (Fig. 4d), freezing was low before the first trial and increased across the conditioning trials (main effect of trial: F(4, 196) = 213.68, P < 0.0001); there were no other significant main effects or interactions (F < 2.09, P > 0.17). Next, rats underwent a retrieval session in which they were presented with the FW or BW CS to reactivate the fear memory and, immediately thereafter, received an intra-dHPC infusion of the protein synthesis inhibitor rapamycin (Rapa; 1.5 μg per side) or Veh, and were then returned to their home cages. During the reactivation session (‘reactivation’; Fig. 4d), FW and BW groups differed in their levels of conditioned freezing. Repeated-measures ANOVA revealed a main effect of trial (F(1, 49) = 115.5, P < 0.0001), a main effect of conditioning procedure (F(1, 49) = 8.36, P = 0.006) and a significant trial × conditioning procedure interaction (F(1, 49) = 23.2, P < 0.0001). Post hoc analyses revealed that although there were no differences within the FW and BW groups (P > 0.31), rats conditioned to a FW CS showed increased average levels of freezing during the retrieval trials relative to groups conditioned to a BW CS (P = 0.0003).

Forty-eight hours later, freezing to the conditioning context was assessed in a 20-min test session. As shown in Fig. 4d, post-retrieval Rapa infusions into the dHPC impaired contextual freezing in BW-, but not in FW-conditioned, rats relative to Veh-treated controls. Repeated-measures ANOVA revealed a significant main effect of time (F(19, 931) = 2.72, P = 0.0001), a significant time × conditioning procedure interaction (F(19, 931) = 2.42, P = 0.0006) and, importantly, a significant conditioning procedure × drug group interaction (F(1, 49) = 6.44, P = 0.01). Post hoc analyses indicated that Rapa reduced freezing in rats when administered after presentation of the BW (P = 0.006), but not FW, CS (P = 0.52) relative to Veh-treated rats. Thus, presentation of the BW CS covertly retrieved a contextual fear memory that was sensitive to HPC protein synthesis inhibition. Importantly, this experiment demonstrates that contextual fear memory could be indirectly reactivated and attenuated without exposure of animals to the conditioning context. This suggests that therapeutic strategies that rely on indirect retrieval in a clinical setting may be viable therapeutic options for inhibition of pathological fear.

Discussion
Here we combined an innovative implementation of a classic behavioral procedure (BW conditioning) to investigate whether indirectly retrieved contextual fear memories within the HPC could be targeted and manipulated. We show that a fear response to a BW CS is mediated through the conditioning context and recruits HPC neurons to a greater degree than a FW CS. We also found that exposure to the indirect CS reinstated conditioning-related activity in a HPC ensemble. Moreover, HPC ensembles retrieved by the BW CS could be captured using activity-dependent expression of DREADDs and pharmacologically reactivated to drive freezing in a context never paired with shock. Lastly, we observed that intra-HPC protein synthesis inhibition disrupted the reconsolidation of a contextual fear memory retrieved covertly by the BW CS. In sum, our work describes HPC representations for covertly retrieved memories and provides novel evidence that HPC engrams reactivated by covert retrieval cues are sensitive to protein synthesis inhibition.
Previous studies employing activity-dependent labeling strategies have shown that the reactivation of contextual fear engrams within the HPC is both necessary and sufficient for the expression of contextual fear.\textsuperscript{18–21} However, in contrast to the current work, these studies captured HPC ensembles during conditioning. Although this has been fundamental to our understanding of processes underlying memory encoding and retrieval\textsuperscript{22–24}, it does not inform clinical interventions for pathological fear memories in individuals that have previous histories of trauma. Accordingly, a critical question is whether retrieval methods used to facilitate episodic recollection of trauma in a clinical setting result in the reactivation of neuronal populations that encoded the initial trauma. This is particularly relevant to studies of reconsolidation, in which neural manipulations target the physical memory trace. Here we show that covert retrieval of a contextual fear memory results in the reactivation of a contextual fear engram and that the chemogenetic activation of this ensemble supports conditioning-related behavior in a neutral context. Moreover, reconsolidation of this indirectly retrieved memory could be disrupted by hippocampal protein synthesis inhibition. Thus, a critical finding from the current study is that indirect retrieval of a contextual fear memory permits the reactivation and attenuation of a HPC engram representing that memory.

Although our results suggest that clinical interventions that rely on indirect retrieval methods (such as imaginal exposure) may be effective for opening a window to modify, edit or erase neural representations of unwanted traumatic fear memories, an important question is whether indirectly reactivated memories are sensitive to anamnesis agents during reconsolidation\textsuperscript{25}. Given that memories integrate into complex associative structures (including outside the HPC), it is unclear whether the reactivation of one element of the associative network results in the reactivation of other parts in a way that renders them sensitive to reconsolidation manipulations. Indeed, a previous study using second-order conditioning procedures with discrete CSs found that directly—but not indirectly—reactivated fear memories undergo reconsolidation within the amygdala\textsuperscript{26}. However, here we report that reconsolidation of an indirectly retrieved contextual fear memory is disrupted by HPC protein synthesis inhibition. This is consistent with previous work showing that presentation of a trace-conditioned CS also renders an associated contextual fear memory sensitive to HPC protein synthesis inhibition\textsuperscript{27}. Although we did not explore whether amygdala protein synthesis is necessary for reconsolidation of fear to a BW CS, these results suggest that the HPC may have a privileged role in this process, which is consistent with its proposed role in episodic memory.

Lastly, although the ultimate goal of reconsolidation-based therapies is to erase traumatic memories, several studies have demonstrated that retrograde amnesia produced by protein synthesis inhibitors is either transient or recoverable\textsuperscript{28–31}. For instance, a recent study found that systemic administration of a protein synthesis inhibitor after a contextual fear conditioning resulted in robust impairments in the expression of that memory that could be recovered by artificial (for example, optogenetic) activation of the contextual fear engram within the HPC\textsuperscript{32,33}. Based on these results, the authors suggest that although the time-limited protein synthesis following learning is dispensable for memory storage, it may be required for effective memory retrieval processes. Although we found that intra-HPC Rapa impaired reconsolidation of a covertly retrieved context memory, it is possible that this reflects a retrieval deficit as opposed to memory erasure. Indeed, recent reports have challenged the idea that contextual and auditory fear memories in rats undergo protein synthesis-dependent reconsolidation\textsuperscript{34,35}. Indeed, we observed spared freezing in Rapa-treated rats during the early portions of the context test in the current study (Fig. 4d). However, it is possible this reflects an incomplete attenuation of protein synthesis within the dHPC or sparing of engram ensembles outside of the dHPC (including extra-HPC regions). Whether this is true for older memories that are less dependent on the HPC\textsuperscript{10,24} is an important avenue for future work.

In conclusion, our results reveal that indirect retrieval of a contextual fear memory results in a labile memory trace in the HPC that is vulnerable to disruption. This process may contribute to the efficacy of clinical interventions, such as imaginal exposure, that rely on indirect retrieval and manipulation of traumatic memories. Developing retrieval-based behavioral or neural interventions that target HPC ensembles may prove particularly effective in attenuating traumatic fear memories in humans.

Online content
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**Methods**

**Subjects.** Adult experimentally naive male Long–Evans rats (200–240 g on arrival, 10–12 weeks old) were obtained from a commercial supplier (Envigo) and used for all experiments. Rats were individually housed in clear plastic cages on rotating racks in a climate-controlled vivarium with a fixed 14/10-h light/dark cycle (lights on at 7:00), and were given access to standard rodent chow and water ad libitum. All experiments were conducted during the light phase. On arrival, all rats were handled by the experimenter (~30 s per rat d−1) for a minimum of 5 days before the start of any surgical or behavioral procedures. All experimental procedures were conducted in accordance with the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and were approved by the Texas A&M University Institutional Animal Care and Use Committee.

**Viruses and drugs.** Plasmids were a generous gift from the laboratory of S. Tonegawa and were packaged at the University of Pennsylvania Vector Core. From these plasmids, and using the activity-dependent cell-labeling experiments, rats received a 50:50 viral cocktail containing AAV9-TRE-hM3Dq-mCherry-RBP (titer: ≥2×1010 genomic copies ml−1) and AAV9-Cre-tTA-BGH (titer: ≥5×1010 genomic copies ml−1) as described above. Clozapine-N-oxide was provided by the Chemical Synthesis and Drug Supply Program of the National Institute of Mental Health. Rapamycin was obtained from LC Laboratories. Doxycycline-containing rodent chow (DOX, 40 mg kg−1) was obtained from Envigo. For the tagging experiments (described below), subjects were fed the DOX diet for at least 10 days before any surgical procedure.

**Surgeries.** For all surgeries, rats were anesthetized with isoflurane (5% for induction, 1–2% for maintenance) and placed in a stereotaxic frame (Kopf Instruments). The hair on the scalp was shaved, povidone-iodine was applied to the skin and a small incision was made in the scalp to expose the top of the skull. The skull was leveled by placing bregma and lambda in the same horizontal plane.

For experiments involving activity-dependent cell labeling, rats received bilateral infusions of the viral cocktail (described above) into the dHPC (same coordinates as above; 700 nL of total infusion volume per hemisphere) using a microinfusion pump (KD Scientific). Specifically, 10-μl syringes (Hamilton) were mounted on the microinfusion pump; polyethylene tubing (no. PE-20, Brain Tree Scientific) connected the syringe to stainless steel injection needles (26-gauge) that were backfilled with the viral cocktail immediately before injection. Virus was infused at a rate of 100 nL min−1 and injector tips were left in the brain for five additional minutes to allow for diffusion. After the infusion procedure, the incision was closed with sutures and postoperative procedures were conducted as described above. Rats were given a 2-week recovery period after surgery and before behavioral testing, to allow for the development of viral infection.

For experiments involving intracranial microinjections of Rapa, small holes were drilled in the skull for placement of two to three anchoring screws. Bilateral stainless steel guide cannulae (5 mm, 26-gauge; Plastics One) were inserted into the dHPC at the following coordinates (relative to bregma): anteroposterior from the behavioral testing room to an adjacent infusion room and the dummy chamber to allow for diffusion of drug, and rats were immediately transported back to their home cages following the infusion process.

**Behavioral apparatus.** All behavioral experiments were conducted in two distinct rooms within the laboratory. Each room housed eight identical rodent conditioning chambers (30 × 24 × 21 cm; Med Associates). Each chamber was housed in a larger external sound-attenuating cabinet and consisted of two aluminum sidewalls and a rear wall, ceiling and hinged front door made from Plexiglas. The grid floor consisted of 19 stainless steel rods wired to a shock source and solid-state grid scrambler for delivery of the footshock US (Med Associates). Each chamber contained a 15-W house light and ventilation fan to provide ambient background noise (~60 dB). Digital cameras were mounted above each chamber for visual recording and observation of behavior. Cues were manipulated to generate three distinct contexts. For context A, the house lights were turned off and overhead white lights and ventilation fans turned on. Cabinet doors remained open for the duration of each session. Chambers were wiped with 1.0% ammonium hydroxide before each behavioral session. Rats were transported to context A in black plastic boxes. For context B, house lights were turned on, fans were turned off and the room was dimly lit by overhead fluorescent red lights. Cabinet doors remained closed for the duration of each behavioral session. Black Plexiglas floors were placed over the grid, and each chamber was wiped down with a 3.0% acetic acid solution before each behavioral session. Rats were transported to context B in white plastic boxes with a clean layer of bedding. For context C, both the house lights and overhead white lights were turned on, fans were turned on and cabinet doors remained open. Chambers were wiped with 70% ethanol before each behavioral session, and rats were transported to context C in white plastic boxes with a clean layer of bedding.

For unbiased measurements of freezing behavior, each behavioral chamber rested on a load-cell platform used to detect chamber displacement in response to each rat’s motor activity. During behavioral testing, load-cell values (ranging from −10 to +10 V) were recorded and digitized at 5 Hz using Threshold Activity software (Med Associates). These values were then transformed to generate absolute values ranging from 0 to 100, with lower values indicating less cage displacement. Freezing was quantified by computing the number of observations for each rat that had a value less than the freezing threshold (load-cell values of 10 or less) for a minimum of five consecutive observations (1 s or more).

**Histological procedures.** Following completion of the experiment, rats were overdosed with sodium pentobarbital (Fatal Plus; 100 mg ml−1, 0.5 ml intraperitoneally) and perfused transcardially with physiological saline followed by 10% formalin. Brains were extracted and stored overnight (at 4 °C) in 10% formalin, after which they were transferred to a 30% sucrose solution for a minimum of 3 days. After fixation and cryoprotection, brains were flash-frozen on dry ice and sections collected using a cryostat (Leica Microsystems) at −20 °C. To verify the activity-dependent expression of hM3Dq-mCherry, coronal sections underwent fluorescent immunostaining (see below) to visualize the localization and intensity of the mCherry expression in the dHPC.

For behavioral experiments involving c-Fos quantification (but in the absence of surgical procedures), coronal sections (40 μm) containing dHPC were collected into well plates containing 1× PBS (pH 7.4) with 0.01% sodium azide and stored at 4 °C until immunohistochemistry was performed. Identical procedures were used for experiments involving viral manipulations, except that 30-μm coronal sections of dHPC were collected.

For cannula experiments, coronal sections (40 μm) were dried on subbed microscope slides and stained with thionin (0.25%) for cannula tract visualization. Specifically, tissue slides were submerged for 5 min each in 95% and 100% EtOH, followed by 10 min of submersion in CitriSolv (Fisher Scientific). Mounted tissue was then submerged in 100% ETOH (3 min), 95% ETOH (2 min), 70% ETOH (2 min) and distilled water (2 min), followed by 0.25% thionin for ~15 s. Tissues were then rinsed in distilled water followed by submersion in 70% ETOH and 0.01% acetic acid (1 min), 70% ETOH (1 min), 95% ETOH (2 min, twice) and 100% ETOH (2 min, twice) before submersion in CitriSolv for 10 min before coverslipping. Glass coverslips mounted on slides using Permount mounting medium (Fisher Scientific), and coronal sections were imaged at x10 using a Leica Microsystems (MZFLIII) with Leica Firecam software.

**Immunohistochemistry.** For detection of c-Fos by immunohistochemistry, slices were first rinsed three times in Tris-buffered saline (TBS, 1×, pH 7.4). All rinses were performed at room temperature on a plate shaker. Tissue slices were transferred across wells using mesh well inserts and then placed in 0.3% H2O2 (in TBS) for 15 min followed by three rinses in TBS. Sections were then incubated overnight in primary antibody (rabbit anti-c-Fos, 1:10,000; Millipore, no. ABE457 (Antibodyregistry.org: AB_2631318)) in TBS containing Tween-20 (TBST). The next day, sections were rinsed three times in TBS and then transferred to secondary antibody for 1 h (biotinylated goat anti-rabbit, 1:1,000; AB_200751)). After three further rinses in TBS, tissues were incubated in avidin biotin complex (ABC; 1:500 in PBS) at room temperature and on a plate shaker unless stated otherwise. Tissues were then rinsed again in TBS three times and then placed in 10% normal donkey serum (NDS) in PBST for 1 h. All steps occurred at room temperature and on a plate shaker unless stated otherwise. Tissues were transferred using mesh well inserts. Slices were then incubated with one or more primary antibodies (1:500 dilution in PBS) at room temperature for 24 h (guinea pig anti-c-Fos, 1:1,000; Synaptic Systems, no. 226 005 (Antibodyregistry.org: AB_2800522) and rabbit anti-β-APP, 1:1,000; Animal Biotechnologies, no. 20085222 and 2007751). The next day, slices were rinsed again in PBST three times and incubated with one or more secondary antibodies (1:500 dilution in PBS) for 2 h at room temperature in 1% NDS in PBST (Alexa Fluor 488 donkey anti-guinea pig; Jackson ImmunoResearch, no. 706-545-148 (Antibodyregistry.org: AB_2340472) and Cy3
Effect of BW CS exposure on c-Fos activity in a HPC fear engram. All rats (n = 14, before exclusions) were given a 20-min exposure session to what would be the retrieval context (B). After this session, all rats were taken off DOX and 48 h later received BW conditioning in context A as described above. Immediately after conditioning, animals were placed back on the DOX diet to prevent further labeling. Twenty-four hours later, half of the rats were randomly assigned to receive five CS-only presentations while the other half were simply exposed to the same context for an identical amount of time. Note that groups were run in different (alternating) squads. Ninety minutes after the first CS presentation of the retrieval session, rats were killed for quantification of the hippocampal activity engram. mCherry+ and co-labeled cells for each image were averaged and divided by the surface area (standardized to 0.1 mm²), unless stated otherwise.

Chemogenetic activation of a covertly captured HPC neuronal ensemble. After an exposure session (day 1), all rats (n = 64, before exclusions) received BW conditioning (day 2) and 48 h later were given a retrieval session, in which they were presented with the BW CS to label and capture putative engram cells in the dHPC (day 5). The next day we examined the impact of chemogenetic engram cell activation on freezing responses in a novel context during a 10-min test session (day 6).

For the exposure session, rats were transported from the vivarium and placed in context B for 20 min; no additional stimuli were presented during this session. This session was conducted in an effort to bias cell labeling during the subsequent capture session to the BW CS presentation, rather than context B itself. The next day, rats received BW conditioning as described above. Immediately after conditioning, rats were taken off DOX (replaced with standard chow) for 4 h to open a labeling window for cell tagging. In addition, we included a control group that remained on DOX throughout the duration of the experiment (OnDOX); note that all rats were randomly assigned to the experimental and control groups before the start of behavioral testing. For the activity-dependent capture session, groups of rats were placed in context B and, after a 3-min BL period, received five CS presentations each separated by a 60-s ISI (Ret). Rats remained in the chamber for 1 min after the last CS presentation, at which time they were returned to their home cages. A control group was included that was exposed to context B for an equivalent amount of time, but did not receive any CS presentations (NoRet). After being returned to their home cages, all rats were immediately placed back on the DOX diet to prevent further cell labeling. Twenty-four hours after cell labeling, rats were injected with either CNO (3 mg kg⁻¹, intraperitoneally) or Veh and placed in a novel context (C) to assess whether reactivation of the tagged BW CS engram was sufficient to drive conditioned freezing. One day after the initial testing, a random subset of rats from each group was killed for quantification of c-Fos and mCherry expression (Ret-CNO, n = 5; Ret-CNO-OnDOX, n = 5; Ret-Veh, n = 5; NoRet-CNO, n = 5). In addition, histological verification of activity-dependent hM3Di-mCherry expression in all rats was performed as described above.

During the experiment, one rat became ill and was immediately euthanized (Ret-CNO, n = 1), and any animal (aside from OnDOX animals) that did not exhibit bilateral expression of mCherry in the dHPC was excluded from the analysis (Ret-CNO, n = 4; Ret-Veh, n = 4; NoRet-CNO, n = 2). Lastly, several rats in the NoRet group (n = 4) exhibited high levels of freezing behavior (>25%) during the capture session, suggesting that contextual fear had generalized to the removal normalac. These animals were removed from the analyses to ensure that we did not inadvertently capture a generalized context fear memory in the NoRet animals. This resulted in the following final group numbers for the behavioral experiment: Ret-CNO, n = 12; Ret-Veh, n = 11; Ret-CNO, n = 9; Ret-CNO-OnDOX, n = 8. This behavioral experiment was performed in two replications with similar outcomes in each, and these were therefore combined for statistical analysis.

Inhibition of protein synthesis in the dHPC after retrieval of FR or BW CS. In a 2 X 2 design, rats (n = 64, before exclusions) were randomly assigned to receive either BW or FR conditioning at testing, or no CS retrieval at test (NoTest). The NoTest group was divided such that half of the rats in that group received BW conditioning while the other half received FR conditioning. One rat was excluded from the analysis due to tissue quality. This resulted in the following group numbers: FR (n = 7), BW (n = 8), NoTest (BW, n = 4) and BW-conditioned (n = 4).

One day before conditioning, all rats were given a 15-min exposure session to what would be the retrieval context (context B). For conditioning, all rats (in squads of eight, groups intermixed) were transported to context A and received either BW or FR conditioning as described above. Twenty-four hours after conditioning, rats in the FW and BW groups were transported from the vivarium to a neutral context (B) and, after a 3-min BL period, were presented with four CS-alone trials. Each CS presentation was separated by a 60-s ISI, with the rat remaining in the chamber for 1 min after the last CS presentation before being transported back to the vivarium. Rats were perfused for 90 min after the first CS of the test. Rats in the NoTest group (with FW- and BW-conditioned animals intermixed) were not given a CS retrieval session, but were perfused alongside groups of rats in the FW and BW groups.
and off-target cannula placements outside of the dHPC (FW-Veh, n = 1; BW-Rapa, n = 1; BW-Veh, n = 1) resulted in the following group numbers: FW-Veh, n = 15; FW-Rapa, n = 13; BW-Veh, n = 14; BW-Rapa, n = 11. Note that one additional rat in the BW-Rapa group was marked as an outlier (±2 s.d. from the group mean) during the context test and was removed from analysis (the above group sizes reflect this).

For conditioning, rats were transported from the vivarium to context A and received either FW or BW conditioning in alternating squads; chambers were counterbalanced for drug assignments in all sessions. Twenty-four hours after conditioning (day 2), rats were given a 20-min exposure session to the retrieval context (B) in the absence of the CS or the US. This exposure session was conducted to reduce any fear that may have generalized across contexts, and to ensure that drug manipulations following the subsequent retrieval session were molecular events associated with reconsolidation of the CS-evoked memory. After exposure (later that same day), FW and BW rats (intermixed in each squad) were returned to the retrieval context (B) and presented with a single CS after a 3-min BL period. The rats remained in the chamber for 1 min (250 s for the entire session), after which they were immediately transported to an adjacent room and received intra-DG infusions of either Rapa or Veh. Rats were returned to their home cages immediately after the infusion process.

Forty-eight hours after drug infusion, rats were returned to the conditioning context (A) for a 20-min context test. No additional stimuli were presented during this session, and rats were transported to the vivarium following the conclusion of the test. Note that this behavioral experiment was performed in two replications with similar outcomes in each, and were therefore combined for statistical analysis.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support the findings of this study are available from the corresponding author upon request. Source data are provided with this paper.

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Author contributions
R.L.R., T.D.G. and S.M. designed the experiments, analyzed data and wrote the manuscript. R.L.R. and T.D.G. collected data for all experiments. S.K. assisted with c-Fos data collection and with the behavioral experiments shown in Fig. 1. K.R.R. assisted with the behavioral experiments shown in Fig. 3.

Competing interests
The authors declare no competing interests.

Additional information
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Freezing values were collected automatically using Threshold Activity Software (Med Associates, V 3.5.1.2). All fluorescent images (including DAB visualization) were taken on a Zeiss microscope using Axio Imager software (Zen Pro 2012, V 1.1.2.0). For cannula experiments, imaging was conducted using a Leica Microscope (MZFLIII) with Leica Firecam software (V 3.4.1). Image J (NIH, V 1.52) was used for histological analysis/cell counting which was conducted manually.

Data analysis

Data were analyzed using conventional parametric statistics (Statview; SAS Institute)

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
No statistical test was used to predetermine group sizes. Group sizes were selected based on what is common in the field and are similar to those reported in previous publications (see methods).

**Data exclusions**
Effects of CS exposure on c-Fos activity in the dHPC: One rat was excluded due to poor tissue quality (FW, n = 1).

Impact of CS exposure on c-Fos activity in a dHPC fear engram: Two rats were excluded due to poor viral infection/tissue quality (Ret, n = 1; NoRet, n = 1).

Chemogenetic activation of a BW-tagged fear ensemble: One rat became ill and did not finish the experiment (Ret-CNO, n = 1). Any rat that did not have bilateral expression of virus was excluded (Ret-CNO, n = 4; Ret-Veh, n = 4; NoRet-CNO, n = 2). Several rats on the NoRet-CNO control group exhibited high levels of freezing (> 25% average for entire session) in the neutral tagging context and were excluded to ensure CNO manipulations were specific to activation of the BW-tagged ensemble, as opposed to a generalized fear memory (NoRet-CNO, n = 4). While this was not preestablished criteria, the experimental design was intended to limit freezing in this group (we included a preexposure session to specifically try and reduce NoRet freezing). Upon completion of the experiment it was apparent that a small subset of rats froze >25% for the session, which is much greater than what would be expected during exposure to a non-shock associated context.

Inhibition of protein synthesis in the dHPC following retrieval of a FW or BW CS: Two rats lost their headcaps and were removed from experiment (FW-Rapa, n = 1; BW-Rapa, n = 1). Three rats were removed from the experiment because of illness (FW-Rapa, n = 1; BW-Rapa, n = 1; BW-Veh, N = 1). Two rats were excluded due to experimenter error during the infusion process (FW-Rapa, n = 1; BW-Rapa, n = 1; BW-Veh, n = 1). Lastly, three rats were excluded due to off-target cannula placements (FW-Veh, n = 1; BW-Rapa, n = 1; BW-Veh, n = 1).

**Replication**
Several of the behavioral experiments (Fig. 3a-b, Fig. 4a-d) were performed in two replications with similar outcomes in each and were therefore combined for statistical analysis.

**Randomization**
All animals were randomly assigned to experimental groups upon arrival. All cages positions in the vivarium were counterbalanced for group assignment. During behavioral testing all group assignments were counterbalanced for chamber position. Forward and Backward conditioned rats were run in alternating squads.

**Blinding**
Analysis of freezing behavior was collected using automatized and unbiased Threshold Activity Software (described above). All imaging and cell counting was conducted with experimenters blind to group assignments.

Reporting for specific materials, systems and methods

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### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☐   | Antibodies |
| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology and archaeology |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |
| ☑   | Clinical data |
| ☑   | Dual use research of concern |

### Antibodies

| Antibodies used | Anti - RFP (Rabbit):1:500, Rockland antibodies and assays, Product Code 600-401-379, Lot # 42722 (Antibodyregistry.org: AB_2209751) |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChiP-seq |
| ☑   | Flow cytometry |
| ☑   | MRI-based neuroimaging |
| Antibody Description                                      | Dilution | Supplier | Product Code | Lot Number |
|-----------------------------------------------------------|----------|----------|--------------|------------|
| CY3-conjugated Anti-Rabbit (Donkey)                       | 1:500    | Jackson Laboratories | 711-165-152 | 143560     |
| Anti-c-Fos (Rabbit)                                       | 1:10,000 | Millipore | ABE457       |            |
| Anti-c-Fos (Guinea Pig)                                   | 1:500    | Synaptic Systems | 226 005, Lot2-6/2-7 | AB_2800522 |
| Alexa Fluor 488 Anti-Guinea Pig (Donkey)                  | 1:500    | Jackson Laboratories | 706-545-148 | 143798     |
| Biotinylated goat anti-rabbit 1:1000                     |          | Jackson Laboratories | 111-065-003 |            |

**Validation**

All antibodies are widely used and we defined optimal antibody dilution by performing serial dilutions when necessary, including negative controls omitting the primary and secondary antibody. All antibodies are referenced in the Antibodyregistry.org (listed above).

**Animals and other organisms**

| Category                  | Description                                                                 |
|---------------------------|-----------------------------------------------------------------------------|
| Laboratory animals        | Rattus Norvegicus, Long-Evans, Male, ~10-12 weeks old. Rats were single housed in a climate controlled vivarium with a fixed 14/10 hour light cycle with ad libitum access to food and water. All behavioral experiments were conducted between 8:00 am and 5:00 pm. |
| Wild animals              | This study did not involve wild animals.                                     |
| Field-collected samples   | This study did not involve field-collected samples.                         |
| Ethics oversight          | All procedures were conducted in accordance with the US National Institutes of Health Guide and Care for use of Laboratory Animals and were approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC). |

Note that full information on the approval of the study protocol must also be provided in the manuscript.