Cortico-hippocampal schemas enable NMDAR-independent fear conditioning in rats

Citation for published version:
https://doi.org/10.1016/j.cub.2018.07.037

Digital Object Identifier (DOI):
10.1016/j.cub.2018.07.037

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Current Biology

Publisher Rights Statement:
This is the author's peer-reviewed manuscript as accepted for publication.

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Title: Cortico-hippocampal schemas enable NMDAR-independent fear conditioning in rats

Authors: Peter S. B. Finnie¹,², Karine Gamache¹, Maria Protopoulos¹, Elizabeth Sinclair¹, Andrew G. Baker ¹, Szu-Han Wang³,⁴, Karim Nader¹,⁴,⁵.

Author Affiliations:

¹Psychology Department, McGill University
1205 Avenue Dr. Penfield
Montreal, QC, Canada
H3A 1B1

²Current address: Picower Institute for Learning and Memory
Massachusetts Institute of Technology
77 Massachusetts Avenue, #46-3301
Cambridge, MA, USA
02139

³Centre for Clinical Brain Sciences
University of Edinburgh
49 Little France Crescent
Chancellor’s building GU507c
Edinburgh, UK
EH16 4SB

⁴Senior author

Address for Correspondence:
s.wang@ed.ac.uk
karim.nader@mcgill.ca

A final version will be published in: Current Biology

DOI: https://doi.org/10.1016/j.cub.2018.07.037

Keywords: hippocampus; anterior cingulate cortex; memory maintenance; fear conditioning; NMDA-receptor; schema; learning and memory; systems consolidation; memory reconsolidation; experience-dependent plasticity.
HIGHLIGHTS

- Fear conditioning is NMDAR independent when the procedure matches prior training
- Hippocampus-dependent memory of prior training is required for NMDAR independence
- However, a reminder cue reinstates NMDAR independence after hippocampal disruption
- Memory maintained by the anterior cingulate cortex enables this mechanistic switch

IN BRIEF

Finnie et al. reveal that contextual fear learning is independent of dorsal hippocampal NMDA receptors in rats previously exposed to a similar conditioning procedure, but not to the training environment. Hippocampus-dependent memory engages this mechanistic switch, yet anterior cingulate cortex maintains the requisite procedural representation.
SUMMARY

The neurobiology of memory formation has been studied primarily in experimentally-naive animals, yet the majority of learning unfolds on a background of prior experience. Considerable evidence now indicates that the brain processes initial and subsequent learning differently. In rodents, a first instance of contextual fear conditioning requires NMDA receptor (NMDAR) activation in the dorsal hippocampus, but subsequent conditioning to another context does not. This shift may result from a change in molecular plasticity mechanisms, or in the information required to learn the second task. To clarify how related events are encoded it is critical to identify which aspect of a first task engages NMDAR-independent learning, and the brain regions that maintain this state. Here we show in rats that the requirement for NMDARs in hippocampus depends neither on prior exposure to context nor footshock alone, but rather on the procedural similarity between two conditioning tasks. Importantly, NMDAR-independent learning requires the memory of the first task to remain hippocampus-dependent. Furthermore, disrupting memory maintenance in the anterior cingulate cortex after the first task also reinstates NMDAR-dependency. These results reveal cortico-hippocampal interactions supporting experience-dependent learning.
Introduction

Pioneering theories of memory formation hold that prior experience strongly influences the learning process [1-3], yet a majority of modern neurobiological studies focus on experimentally-naive organisms. For tasks thought to rely on hippocampus, different mechanisms can mediate encoding of new experiences and subsequent similar events [4-17]. In rats the pre-training infusion of competitive N-Methyl-D-aspartate receptor (NMDAR) antagonist, DL-(2R)-amino-5-phosphonovaleric acid (AP5) into the dorsal hippocampus (dHC) impairs fear conditioning to a first but not a second novel context [6, 7, 9]. Thus, Training$_1$ is NMDAR-dependent (AP5-sensitive) and Training$_2$ is NMDAR-independent (AP5-insensitive).

This mechanistic switch could reflect two distinct processes. Repetition of information could obviate the need for plasticity in the neural circuits that already encode the redundant experience (i.e. assimilation). Alternatively, prior learning could alter the cellular mechanisms required to induce additional plasticity in the same circuit (i.e. homeostatic plasticity). Disambiguating these processes demands an understanding of the mnemonic representations capable of eliciting NMDAR-independent learning, along with the brain systems that mediate these requisite memories. Prior studies have approached these questions using a variety of hippocampus-dependent behavioral tasks, but have not reached consensus. For instance, blocking cellular consolidation in dHC after Training$_1$ prevents NMDAR-independent contextual fear conditioning [7], suggesting that a representation of the spatial context may be sufficient to drive the switch [18]. Indeed, exposure to a training environment can subsequently engage NMDAR-independence [8, 10, 19], albeit with notable exceptions [4, 15, 20]. Debate also lingers regarding whether NMDAR-activity remains necessary in brain regions outside of dHC [9, 19, 21] or is relieved brain-wide [4, 8, 22]. Even studies reporting that contextual pre-exposure engages NMDAR-independent learning
Authors’ pre-print version

diverge on whether this reflects a change in the anatomical sites [19] or molecular mechanisms [8, 22] of plasticity. Thus any instance of NMDAR-independent learning may result from a combination of factors related to the experimental methodology and nature of information redundancy animals encounter across multiple behavioral experiences.

Using convergent approaches, here we characterize in rats the conditions that render contextual fear conditioning insensitive to AP5 infused into dHC. We set out to test whether it is the representation of the context, shock, or context-shock association encoded during an initial fear conditioning episode that engages NMDAR-independent mechanisms. Instead we observe that the mechanistic switch occurs only when the two conditioning procedures share a similar arrangement. Surprisingly, these NMDAR-dependent and -independent learning protocols elicit comparable activation of hippocampus but differential activation of anterior cingulate cortex (ACC), motivating us to manipulate memory retention in these regions during the inter-training interval. Reducing the reliance of Training1 memory on hippocampus restores sensitivity to dHC-infused AP5, but a reminder of the Training1 context reinstates NMDAR-independent mechanisms. The reminder may re-establish access to memory of the conditioning procedure retained within the ACC, as disrupting memory maintenance in this structure after Training1 prevents NMDAR-independent learning.

Our approach deconvolves each region’s contribution to retention of Training1 memory from the formation of Training2 memory. Together, our findings suggest that the hippocampus transiently links to neocortical representations of a prior episode, which can switch the plasticity mechanisms recruited to encode another procedurally-similar task. The ACC is inferred to mediate abstract statistical regularities extracted across two similar events, precluding the requirement for dHC NMDARs while learning the second task.
Results

Identifying the components of Training\textsubscript{1} that engage AP5-insensitive learning.

To explore how the brain forms contextual fear memories within a background of prior experience, we first aimed to identify the mnemonic components of an initial learning event that can subsequently engage AP5-insensitive mechanisms. We began by replicating protocols used in prior studies [6, 7] to confirm that a second instance of contextual fear conditioning is not disrupted by AP5 infused into dHC (Fig. 1A). We reasoned that the mnemonic representation of any component of this first conditioning episode – the context (conditioned stimulus, CS), footshock (unconditioned stimulus, US), CS-US association, or conditioned fear response (CR) – could be sufficient to subsequently permit AP5-insensitive learning. In a previous study it was demonstrated that Training\textsubscript{2} remains AP5-insensitive even when Training\textsubscript{1} is extinguished [7, 9], thus the ability to express the CR is not required for NMDAR-independent learning. To examine the requirement for representations of the CS, US, or CS-US association, here we systematically modified the Training\textsubscript{1} procedure to determine if rats exposed to the context, to footshock (such that minimal contextual conditioning was induced), or to both independently would subsequently exhibit AP5-insensitive learning during Training\textsubscript{2}. Leading theories postulate that the representation of contextual fear conditioning mediated by the dHC should be functionally equivalent even if the US is omitted [18, 23, 24]. Thus rats experiencing either contextual fear conditioning or the context alone during Training\textsubscript{1} should exhibit AP5-insensitive learning.

Surprisingly, we observed that rats exposed to Context\textsubscript{1} without footshocks during Training\textsubscript{1} do not subsequently show AP5-insensitive learning. To maintain consistency with previous studies, rats received a long-term memory test (LTM\textsubscript{1}) in the same context 24h after Training\textsubscript{1}, during which minimal freezing was observed (Fig. 1B, left). The rats were then
pseudo-randomly assigned to receive bilateral dHC infusions of either AP5 or control vehicle (VEH) immediately prior to Training\(_2\), based on pairs matched for LTM\(_1\) freezing. Training\(_2\) consisted of contextual fear conditioning in a second distinct environment (Context\(_2\)), and was administered 4 days after LTM\(_1\). Critically, rats infused with AP5 before Training\(_2\) froze significantly less during LTM\(_2\) than those infused with VEH. In complementary experiments, freezing during LTM\(_2\) was also reduced in rats treated with AP5 prior to Training\(_2\) despite prior exposure to Context\(_2\) for either a short (270s) or a long (2 x 1,800s) duration (see Fig. S1). These findings indicate that prior exposure to context alone is not sufficient to engage AP5-insensitive learning mechanisms.

We next showed that exposure to the US during Training\(_1\) is not sufficient to engage AP5-insensitive learning mechanisms during Training\(_2\). To minimize the formation of a CS-US association during presentation of the US, rats were given an immediate shock procedure [25, 26]. Specifically, each animal was placed into the conditioning chamber and \(~\)15s later received two footshocks (1s inter-shock interval), before being removed. When returned to Context\(_1\) 24h later for LTM\(_1\), most rats exhibited minimal freezing (Fig. 1C, left side). Those freezing >35% were excluded due to their acquisition of a fear association (\(n = 1\), see Methods and Table S1). Critically, when these previously shocked rats were infused with AP5 prior to Training\(_2\) they froze significantly less during LTM\(_2\) than those given VEH (Fig. 1C, right), suggesting that NMDAR activity remained necessary for learning. Additional groups received Training\(_1\) that consisted of either one or three immediate shocks in Context\(_1\) (Fig. S2) or two immediate shocks in Context\(_2\) (Fig. S3), yet in all cases AP5 prior to Training\(_2\) significantly impaired fear conditioning. Thus, neither the CS nor US alone during Training\(_1\) enables AP5-insensitive fear conditioning during Training\(_2\). Instead, some property of the CS-US association
acquired during Training$_1$ must be necessary for AP5-insensitive fear conditioning during Training$_2$.

We hypothesized that Training$_2$ might remain AP5-sensitive if the procedural arrangement of the two fear conditioning tasks differed substantially. Toward this end, rats received a distinct CS-US association procedure during Training$_1$ (Fig. 1D). Unlike the standard delayed conditioning (DC) protocol used in Fig. 1A (two footshocks delivered 180s after initial placement into the context), in this experiment Training$_1$ consisted of a two-day protocol in which rats were pre-exposed to the chamber during a first session and then given two immediate footshocks in the same context 1 day later. The procedure is referred to as the context pre-exposure facilitation effect (CPFE), and was adapted from protocols developed by Fanselow, Rudy, and colleagues [27, 28]. The nature of the contextual fear associations formed by DC and CPFE procedures have typically been treated as functionally equivalent [18, although see 29-32]. If this is the case, then rats receiving either procedure during Training$_1$ should exhibit AP5-insensitive acquisition of DC during Training$_2$. To test this prediction, following CPFE in Context$_1$ rats were matched based on LTM$_1$ freezing and assigned to receive AP5 or VEH immediately prior to Training$_2$. Both groups exhibited minimal freezing in Context$_2$ prior to footshock delivery (VEH = 4.69±3.39%, AP5 = 1.042±0.705%; Mann-Whitney $U = 20$, $p = 0.57$), indicating little fear generalization from Training$_1$. The day after Training$_2$, each rat was returned to Context$_2$ for the LTM$_2$ test. Strikingly, in these animals for which the Training$_2$ conditioning procedure differed from that experienced during Training$_1$, AP5 blocked fear acquisition (CPFE$\rightarrow$DC; Fig. 1D). Thus, rats successfully acquired Training$_2$ in the presence of AP5 only if they had previously received the same conditioning procedure during Training$_1$ (DC$\rightarrow$DC; Fig. 1A). These results are inconsistent with the argument that the DC and CPFE procedures produce functionally equivalent associative fear memories.
Additional control experiments demonstrated that minor procedural discrepancies did not prevent AP5-insensitive learning. For instance, when the context and footshock exposure sessions from the CPFE procedure were combined into a single DC session lasting 750s during Training₁, rats were subsequently insensitive to AP5 infused prior to the standard DC procedure lasting 270s during Training₂ (Fig. S4). Furthermore, rats given an altered DC procedure during Training₁ that included either more or fewer footshocks could still acquire the standard two-shock DC procedure following AP5 infusions (Fig. S5).

**AP5-insensitive learning depends on the similarity of Training₁ and Training₂ procedures**

One potential interpretation of the previous experiments is that the two training protocols must be similar to engage AP5-insensitive learning. Alternatively, the CPFE procedure could produce a memory that is simply weak or transient. To dissociate these alternatives, we again assigned rats to receive either DC or CPFE during Training₁, but each then experienced CPFE during Training₂. Infusions of AP5 or VEH were given on the second day of Training₂, immediately before the immediate footshock (CS-US association) phase. As hypothesized, rats were impaired by AP5 relative to VEH when Training₁ consisted of DC (Fig. 2A), but not when it consisted of CPFE and thus matched the Training₂ procedure (Fig. 2B). Together, Figs. 1-2 indicate that the brain differentiates the conditioned associations acquired via DC and CPFE procedures. The fear conditioning procedures encountered during each task need to be similar (although not identical) in order to engage AP5-insensitive learning mechanisms.

**AP5-insensitive learning requires the memory of Training₁ to be hippocampus-dependent.**
We have previous reported that infusion of a protein synthesis inhibitor into dHC disrupts the consolidation of Training\(_1\) memory and also AP5-insensitive learning \([7]\). Thus, at the time of Training\(_2\) an intact hippocampally-mediated representation of Training\(_1\) may be critical to engage NMDAR-independent mechanisms. In the weeks following acquisition contextual fear memories gradually transform into a more “gist-like” form \([33]\) that no longer depends on hippocampus for expression \([34, 35]\). Thus we hypothesized that one month after Training\(_1\) the natural loss of mnemonic detail and/or hippocampal-dependence that occurs over time should revert Training\(_2\) to an AP5-sensitive state.

In the first phase of this experiment, Training\(_1\) consisted of a DC procedure followed 1 day later by LTM\(_1\) (Fig. 3, ‘1d’ left side). Unlike in our previous experiments (i.e. Fig. 1A), Training\(_2\) and the preceding VEH/AP5 infusions were administered 30 (rather than 4) days after LTM\(_1\). Both groups exhibited little freezing during the pre-shock interval of Training\(_2\) (1d+VEH = 8.18±3.65%, 1d+AP5 = 4.82±1.86%; Mann-Whitney \(U = 37.0, \ p = 0.549\)), indicating minimal generalization of fear to Context\(_2\) even after the long inter-training interval. During the LTM\(_2\) test 1 day later, AP5-infused rats froze significantly less than those given VEH (Fig. 3, ‘1d’ right side). This tentatively suggests that for Training\(_2\) to remain AP5-insensitive, the Training\(_1\) memory might have to be in a hippocampus-dependent state – a conclusion supported by other recent findings \([22, 36]\).

Previous studies have revealed that expression of a remote contextual fear memory can transiently return to a hippocampus-dependent state in the hours after rats are re-exposed to the conditioning chamber \([37, 38]\), through a process called systems reconsolidation. We hypothesized that reminding the animals of Training\(_1\) the day before Training\(_2\) might likewise reinstate AP5-insensitive learning. Therefore, we gave a second group of rats the LTM\(_1\) test 30 days after Training\(_1\), followed just 1 day later by Training\(_2\). The
LTM\textsubscript{1} test was intended to serve as a reminder to re-engage hippocampal dependency of the Training\textsubscript{1} memory. LTM\textsubscript{1} freezing was equivalent in both drug groups (Fig. 3, ‘30d’ left side), but was significantly higher than in animals tested just 1d after Training\textsubscript{1}. This effect may be attributable to fear incubation [39]. The animals treated with VEH or AP5 before Training\textsubscript{2} exhibited comparably low pre-shock generalized fear during Training\textsubscript{2} (30d+VEH = 9.35±4.66%, 30d+AP5 = 2.23±1.98%; Mann-Whitney $U = 29.5$, $p = 0.340$). Critically, they also froze equivalently during LTM\textsubscript{2} (Fig. 3, ‘30d’ right side). Thus, LTM\textsubscript{1} given just 1d before Training\textsubscript{2} can re-engage AP5-insensitive learning, perhaps via a process like systems reconsolidation, which is postulated to return Training\textsubscript{1} memory expression to a hippocampus-dependent state.

**Disrupting memory maintenance in hippocampus after Training\textsubscript{1} prevents AP5-insensitive learning.**

The previous findings suggest that a hippocampally-mediated representation of Training\textsubscript{1} is required to engage AP5-insensitive learning mechanisms during Training\textsubscript{2}. To confirm this directly we aimed to impair retention of the Training\textsubscript{1} representation specifically in hippocampus by locally infusing peptides known to disrupt the maintenance of long-term potentiation and long-term memory. The peptide pepR845A (pepR) is thought to trigger AMPA-receptor internalization [40, 41], and pilot experiments revealed amnesia when it was infused into dHC 1 day after DC and tested 1 day post-infusion (pepR = 27.35±10.72%, scrambled control peptide = 64.23±10.31%, $t_9 = 2.467$, $p = 0.036$).

If the long-term representation required for NMDAR-independent learning is mediated by the hippocampus, then infusing pepR after Training\textsubscript{1} should render Training\textsubscript{2} sensitive to AP5. To test this hypothesis, rats were given infusions of pepR or scrambled
control peptide (SCR) into both the dorsal and ventral hippocampus (d+vHC) 1 day after Training₁ and were tested 1 day later. We infused into both hippocampal poles to overcome functional compensation between these regions at the time of Training₂ [9]. Rats given pepR froze substantially less during LTM₁ than those given SCR (Fig. 4A, left). Five days after Training₁ these animals then received dHC infusions of AP5 or VEH immediately before Training₂. As anticipated, SCR-treated rats that received either AP5 or VEH exhibited comparably robust freezing during LTM₂ (Fig. 4A, right). Unexpectedly, rats receiving pepR after Training₁ exhibited overall lower freezing levels during LTM₂ than those that had received SCR, indicating a persistent impairment of memory formation or expression (~10-20% reduction in freezing). Nevertheless, the pepR-treated group that exhibited amnesia for Training₁ displayed no evidence of additional impairment of Training₂ caused by AP5, relative to VEH (Fig. 4A, right). Thus, AP5-insensitive learning persists even after disrupting retention of the mnemonic representation of Training₁ in hippocampus.

However, Fig. 3 indicated that a reminder given shortly before Training₂ can re-engage AP5-insensitive learning. Therefore, we reasoned that in Fig. 4A the LTM₁ test administered 24h after pepR infusion might likewise reinstate AP5-insensitive learning by re-engaging the hippocampus. To test this possibility we again infused pepR or SCR into d+vHC 24h after Training₁, but administered no LTM₁ test. As predicted, without LTM₁ serving as a reminder, rats given pepR after Training₁ and AP5 prior to Training₂ froze significantly less than all other groups (Fig. 4B, right). As the pepR+VEH and SCR+VEH groups froze similarly during LTM₂, it is possible that the partial impairment seen in Fig. 4A (right side) only emerges when hippocampal-dependence is reinstated by a reminder. Thus, these experiments indicate that Training₁ memory needs to be in a hippocampus-dependent state to enable AP5-insensitive
acquisition of Training$_2$. Furthermore, re-exposing amnesic rats to the Training$_1$ context can re-establish the AP5-insensitive acquisition of Training$_2$ despite their LTM$_1$ freezing deficit.

**Identifying the brain regions engaged during AP5-sensitive and AP5-insensitive learning.**

The requirement for hippocampus-dependent Training$_1$ memory to engage AP5-insensitive learning suggests that similar and dissimilar tasks may evoke distinct neuronal activity patterns at the time of Training$_2$. To visualize regional brain activation, 90 minutes after Training$_2$ we immunohistochemically labeled neurons expressing the immediate early gene cFos – a marker for recent neuronal activity [42]. Four groups of rats were trained using the procedures described in Figures 1A, 1D, and 2: DC→DC, CPFE→DC, DC→CPFE, and CPFE→CPFE. A fifth group received no training (home-cage control; n = 6). To characterize the effects of task similarity on regional activity, we combined the DC→DC and CPFE→CPFE conditions into a *similar* condition (n = 12), and CPFE→DC and DC→CPFE into a *dissimilar* condition (n = 14). Nuclei positive for cFos were counted bilaterally in three regions of interest (ROIs: CA1 region of dHC, the ACC, and primary somatosensory cortex, S1), selected based on prior studies [43, 44].

Figure 5 shows that both the *similar* and *dissimilar* conditions triggered widespread cFos expression in the ACC, CA1, and S1 when compared to the home-cage group, which is consistent with previous work on schema formation [43]. Rats in the *similar* condition expressed cFos in fewer ACC neurons than those in the *dissimilar* condition, while no difference was observed in CA1 or primary somatosensory cortex (S1). The smaller population of ACC neurons activated following two similar tasks could be due to a phenomenon like repetition suppression [45] or sparsified neural coding [46, 47]. Rats previously fear conditioned using a similar procedure might store this event by recruiting and refining an
already established neuronal ensemble in the ACC, the existence of which could be required to engage AP5-insensitive learning mechanisms.

**Disrupting memory maintenance in ACC after Training\textsubscript{1} does not impair conditioned fear expression yet interferes with AP5-insensitive learning.**

To determine if memory mediated by the ACC is necessary for AP5-insensitive learning, we next infused pepR into this region 24h after Training\textsubscript{1}. The day after infusion we observed no difference in LTM\textsubscript{1} freezing between groups treated with pepR and those given SCR (Fig. 6, left), suggesting either that this peptide has no effect in the ACC or that memories maintained in this region are dispensable for contextual fear expression. In support of the latter position, rats that had received pepR displayed a significant freezing deficit when infused with AP5 before Training\textsubscript{2}, relative to VEH (Fig. 6, right). There was no statistical difference observed between VEH and AP5 groups that had previously received SCR. Thus, the ACC maintains some aspect of the Training\textsubscript{1} memory that is necessary for AP5-insensitive acquisition of Training\textsubscript{2}, but is not required to elicit fear expression during LTM\textsubscript{1}.

**Discussion**

When two contextual fear conditioning episodes share a similar procedural arrangement, the initial training enables NMDAR-independent learning mechanisms to be recruited during the second task. We have demonstrated that facets of memory maintained by hippocampus and ACC are required to engage this experience-dependent state.

Mounting evidence indicates that NMDAR-independent learning does not reflect a unitary experience-dependent phenomenon. We have reported that rats pre-exposed to a conditioning context still require NMDAR-dependent plasticity in dHC to form a context-shock
association (Fig. 2A, S1A-B). These results are consistent with at least one prior study [20], and are bolstered by evidence of place cell remapping [48, 49] and related hippocampal plasticity [30, 50-53] when reinforcing stimuli are administered in a familiar environment. Yet other experiments in rodents indicate that context exposure can be sufficient to lift the requirement for NMDAR activity during fear conditioning [19], even in amygdala [8] where they were previously reported to be indispensible [19, 55]. The dependence on the ACC [36] and effect of prolonged inter-task interval [36, 22] are also inconsistent, likely attributable to the different species, NMDAR-antagonists, routes of drug delivery, and training protocols used. As prior experience can be a nebulous experimental variable across studies, plasticity mechanisms may be profoundly inconsistent depending on the nature of overlap with the task under investigation.

Here, AP5-insensitive learning is engaged only when a second task involves a similar fear conditioning procedure, suggesting that footshock delivery during Training$_2$ reactivates elements of the Training$_1$ memory (Figs. 1-2) [7, 9]. The second task could be encoded by recruiting a neural circuit that overlaps with the first. Reduced ACC activity evoked during a second similar fear conditioning task (Fig. 5) could be due to repetition-suppression [45] or the “sparsification” of the neuronal ensemble [46, 47]. As memory in the ACC must persist to engage AP5-insensitive learning, this region likely contributes to the mnemonic representation of Training$_1$ shared by Training$_2$, albeit not components driving conditioned responding (Fig. 6). Instead, the ACC might encode the temporal properties of conditioning – a function broadly ascribed to dorsomedial prefrontal regions [54, 55]. Disrupting memory maintenance in the ACC might thus alter response timing, for which our task is not sensitive given the high freezing levels. Alternatively, the ACC could serve a more general function by encoding or amplifying representations of regularities detected across multiple episodes.
Hippocampus and neocortex are postulated to make up parallel complementary systems that rapidly encode the trial-specific details of each discrete episode and gradually extract generalities across events, respectively [56, 57]. Indeed, doubling the number of training trials rats experience during a one-day spatial task switches memory retrieval from a hippocampus-to an ACC-dependent state [58]. Although the mnemonic representation stored in our paradigm does not involve a gradual incremental learning process typically used in studies of ‘schema’ formation [43, 44, 59], the experience-dependent changes in learning mechanisms can be conceptualized within the framework of schema-based encoding. Notably, the coordinated recruitment of hippocampus and ACC is implicated in retrieval or updating of schemas during spatial learning tasks [43, 44]. The contribution of hippocampus is diminished albeit necessary during schema-based learning, which may relate to our observation that Training¿ memory must be mediated by hippocampus at the time of Training¿ to engage dHC NMDAR-independent learning (Fig. 4). Speculatively, the ACC may obviate the requirement for dHC NMDARs by selecting contextual features of hippocampally-mediated representations that are temporally predictive across tasks (i.e. grid floor). Even if AP5 impairs the contextual representation formed during Training¿, generalization of specific cues across tasks could sustain normal conditioned responding. Complementary learning systems might enable the brain to incrementally identify particular features across episodes that reliably signal salient outcomes [57, 60-62].

Cellular reconsolidation could underlie updating of the Training¿ memory during Training¿ [50]. Although a second instance of fear conditioning in a distinct context does not render the conditioned response to the first context sensitive to disruption [8, 63], this does not imply that reconsolidation has not been induced. Reconsolidation could feasibly occur in a functionally-delineated manner, hence memory reactivation during a second similar
training procedure might only destabilize common aspects of the trace (i.e. memory for the conditioning procedure) mediated by distinct brain regions (i.e. the ACC), without necessarily disrupting the conditioned response. Indeed, different brain regions [60] and discrete components of interrelated associative memories [64, 65] have been observed to undergo reconsolidation independently. This may even occur within a single region, as dissociable projections from entorhinal cortex to hippocampus have been proposed to mediate the encoding of spatial and non-spatial properties of events [66, 67]. Each may possess distinct properties of NMDAR-mediated transmission [66, 68, 69] and plasticity [70], thus in our task it is conceivable that the former supports conditioned responding and the latter underlies procedural memory, which could be reactivated independently. Even if reconsolidation is not induced, experience-dependent changes in the expression of mechanisms that regulate memory destabilization may dictate which circuits undergo plasticity during related episodes [71].

In Figures 3-4 the hippocampal-dependence of Training₁ memory was putatively reduced, triggering a loss of AP5-insensitive learning that could be recovered by re-exposure to the training context. Synaptic reentry reinstatement models propose that a reminder may strengthen vestigial circuits underlying a disrupted memory, thereby leading to the recovery of the original trace [72]. Context pre-exposure alone does not induce NMDAR-independence (Fig. S1), thus the reminder effect may not rely solely on new learning, but rather rejuvenating inaccessible components of the original memory trace [38, 73, 74]. Indeed, performance of a learned spatial task is impaired following partial hippocampectomy in rats, but accurate navigation can recover following exposure to misleading cues [75]. As recovery is not observed in rats with extensive hippocampal lesions, reminders must either strengthen residual hippocampal representations or re-establish connectivity between
extrahippocampal structures (i.e. the ACC) via spared tissue. Synaptic AMPARs at long-range CA1 projections to ACC [47] may persist after hippocampal pepR infusion. Alternatively, apart from AMPAR internalization, other intrahippocampal synaptic modifications might remain following pepR infusion, including altered expression of NMDARs or other plasticity-induction mechanisms. While a reminder does not trigger recovery of contextual fear response following pepR infusion into dHC [41], it could ‘prime’ these vestigial circuits for encoding [76]. In either case, we theorize that hippocampal physiology is well-suited to recovery based on partial cues. The ACC is either ill-suited or undergoes irreversible pepR-induced amnesia due to a lack of mnemonic redundancy across other regions.

In conclusion, we propose that the brain extracts regularities across two brief episodes, which can cause similar tasks to be encoded via distinct neurobiological mechanisms. Insensitivity to AP5 infused into dHC could reflect a process by which a new contextual fear event is interleaved with existing schematic knowledge about the specific temporal arrangement of conditioning, putatively maintained by neocortical regions including ACC.
Acknowledgements.

The authors would like to thank our past and present lab members for their insightful comments on this study, particularly Virginia Migues for acquisition and preparation of the peptides and Oliver Hardt for his extensive editing of the manuscript. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC; RGPIN 249880-11, SMFSU 373515-09, and RGPIN-249880-06) and Canadian Institutes of Health Research (CIHR; MOP-74672, MOP-93540, and MOP-123430) grants and Royal Society (RG130216, S.-H.W.) grants. P.S.B.F. was the recipient of NSERC Canada Graduate Scholarships – Master’s (CGS-M) and Doctoral (CGS-D) research fellowships.

Author Contributions.


Declaration of Interests.

The authors declare no competing interests.
References


Figure 1. Fear conditioning remains AP5-sensitive after exposure to a distinct context, to footshock, or to context then footshock.

Rats received Training$_1$ consisting of delay conditioning (DC), context exposure, immediate footshock, or Context$_1$ exposure then immediate footshocks on consecutive days (CPFE), and were tested 24h later in the same environment (Context$_1$). Each was then assigned to receive VEH or AP5 immediately prior to DC in Context$_2$ (n/group: DC$\rightarrow$DC+VEH = 7, DC$\rightarrow$DC+AP5 = 8, context$\rightarrow$DC+VEH = 6, context$\rightarrow$DC+AP5 = 6, footshock$\rightarrow$DC+VEH = 8, footshock$\rightarrow$DC+AP5 = 7, CPFE$\rightarrow$DC+VEH = 7, CPFE$\rightarrow$DC+AP5 = 8).
(A) DC in Context\textsubscript{1} produced reliable freezing during LTM\textsubscript{1} that was equivalent in rats assigned to each drug group (to-be-VEH = 68.23±8.41%; to-be-AP5 = 61.49±7.92%; \(t_{13} = 0.17, p = 0.87\)). Rats infused with VEH (68.24±8.41%) or AP5 (61.49±7.92%) prior to Training\textsubscript{2} exhibited statistically comparable freezing during LTM\textsubscript{2} (two-tailed \(t_{13} = 0.584, p = 0.57\)). See also Fig. S5.

(B) Rats exposed to Context\textsubscript{1} for 270s during Training\textsubscript{1} exhibited minimal freezing during LTM\textsubscript{1} (to-be-VEH = 1.39±0.65%; to-be-AP5 = 1.47±0.36%; \(t_{10} = 0.099, p = 0.923\)). Those infused with AP5 prior to Training\textsubscript{2} on average froze significantly less during LTM\textsubscript{2} than those given VEH (VEH = 57.58±5.21%; AP5 = 20.12±6.83%; two-tailed Mann-Whitney \(U = 3, p = 0.015\)). See also Fig. S1.

(C) Animals exposed to 2 immediate shocks in Context\textsubscript{1} during Training\textsubscript{1} likewise exhibited minimal freezing during LTM\textsubscript{1} (to-be-VEH = 2.04±1.61%, to-be-AP5 = 3.05±1.52%, Mann-Whitney \(U = 22, p = 0.51\)). Relative to VEH, AP5 infusion prior to Training\textsubscript{2} impaired freezing during LTM\textsubscript{2} (VEH = 61.12±7.21%; AP5 = 32.21±9.08%; two-tailed \(t_{13} = 2.52, p = 0.026\)). See also Figs. S2-S3.

(D) Rats given CPFE as Training\textsubscript{1} expressed robust freezing during LTM\textsubscript{1} (to-be-VEH = 60.77±10.57%; to-be-AP5 = 57.58±9.38%; \(t_{12} = 0.23, p = 0.83\)), yet mean freezing during LTM\textsubscript{2} was significantly lower for rats that had been infused with AP5 (38.1±8.86%) rather than VEH-(77.71±7.46%) prior to Training\textsubscript{2} (\(t_{12} = 3.263, p = 0.007\)). See also Fig. S4.

Data are expressed as mean percent of time spent freezing during LTM ± s.e.m.
Figure 2. Only rats receiving similar conditioning protocols during Training\textsubscript{1} and Training\textsubscript{2} exhibit AP5-insensitive learning.

(A) Using the DC\textrightarrow CPFE protocol, mean freezing during LTM\textsubscript{1} was statistically equivalent for rats assigned to the to-be-AP5 (48.79±9.06%) and to-be-VEH (55.28±8.42%) groups ($t_{13} = 0.51$, $p = 0.62$). However, rats infused with AP5 prior to Training\textsubscript{2} froze significantly less during LTM\textsubscript{2} on average (26.04±5.73%) than those infused with VEH (59.05±7.97%; $t_{13} = 3.029$, $p = .01$). $n$/group: DC\textrightarrow CPFE+VEH = 9, DC\textrightarrow CPFE+AP5 = 6.

(B) Using the CPFE\textrightarrow CPFE protocol, mean LTM\textsubscript{1} freezing of rats assigned to the to-be-VEH (60.69±9.95%) and to-be-AP5 (67.51±6.61%) rats was not statistically different ($t_{15} = 0.58$, $p = 0.57$). However, mean LTM\textsubscript{2} freezing of rats given VEH (52.71±10.13%) and AP5 (55.35±10.56%) before Training\textsubscript{2} also did not differ ($t_{15} = 0.179$, $p = 0.86$). $n$/group: CPFE\textrightarrow CPFE+VEH = 8, CPFE\textrightarrow CPFE+AP5 = 9.

Data are expressed as mean percent of time spent freezing during LTM test ± s.e.m.
Figure 3. Systems consolidation and reconsolidation of the AP5-insensitive learning state.

(left) Animals tested 1d after Training₁ froze significantly less (to-be-VEH: 44.68±7.42%; to-be-AP5: 40.25±5.62%) than those tested 30d after Training₁ (to-be-VEH: 71.88±6.9%; to-be-AP5: 69.35±5.88%). Two-way ANOVA revealed a main effect of test day (F₁,₃₃ = 18.9, p = 0.0001) but not to-be-infused drug (F₁,₃₃ = .289, p = .595), and also no test day by drug interaction (F₁,₃₃ = .021, p = 0.885).

(right) Two-way ANOVA on LTM₂ freezing revealed a main effect of test day (F₁,₃₃ = 4.853, p = 0.035), drug (F₁,₃₃ = 18.79, p = 0.0001), and an interaction of test day and drug (F₁,₃₃ = 12.85, p = 0.001). Tukey’s posthoc pairwise comparisons indicated that rats tested 30d after Training₁ and infused with either VEH (70.54±5.29%) or AP5 (65.94±7.21%) prior to Training₂ froze comparably during LTM₂ (t₃₃ = .964, p = 0.919). However, AP5-infused rats tested 1d after Training₁ were impaired relative to VEH-treated rats (30.5±5.84% and 78.99±5.62%, respectively; t₃₃ = 5.529, p < 0.0001), but also 30d+VEH (t₃₃ = 4.565, p = 0.0004) and 30d+AP5 (t₃₃ = 4.146, p = 0.0013) groups. n/group: 1d+VEH=9; 1d+AP5=9; 30d+VEH=9; 30d+AP5=10.

Data are expressed as mean percent of time spent freezing during LTM test ± s.e.m.
Figure 4. PepR845A infused into the hippocampus impairs Training$_1$ memory retention, but Training$_2$ is only AP5-sensitive when LTM$_1$ is omitted.

(A, left) A two-way ANOVA on LTM$_1$ freezing revealed a main effect of peptide ($F_{1,33} = 13.337, p = 0.001$), but no main effect of to-be-infused drug ($F_{1,33} = 0.369, p = 0.547$) or interaction of peptide and to-be-infused drug ($F_{1,33} = 0.021, p = 0.885$). Thus rats administered pepR froze significantly less (41.49±6.65%) than those given SCR (72.22±4.97%).

(B) Due to violation of homogeneity of variance (Levene’s $F = 3.3, p = 0.032$), LTM$_2$ freezing scores were assessed via planned Mann-Whitney comparisons with Bonferroni correction (alpha = 0.025). Rats receiving SCR+VEH (median = 82.57%) and SCR+AP5 (median = 80.1%) exhibited equivalent freezing levels during LTM$_2$ ($U = 49, p = 0.7$). However, rats receiving pepR+AP5 froze significantly less (median = 36.63%) than those receiving pepR+VEH.
(median = 89.67%) during LTM$_2$ ($U = 8, p = 0.006$). $n$/group without LTM$_1$: SCR+VEH=11, SCR+AP5=10; pepR+VEH=9; pepR+AP5=8.

Data are expressed as mean percent of time spent freezing during LTM test ± s.e.m.

Figure 5. Neuronal activity evoked following similar versus dissimilar conditioning procedures.

(A) Schematic diagram [78] and representative slices for each ROI from animals in each group. Anterior/posterior distance from bregma inlayed at right. Scale bar represents 200 μm.

(B) Planned Mann-Whitney tests revealed that there were significantly fewer cFos-positive neurons in ACC from rats that had received similar relative to dissimilar training procedures (564.8% and 822.7% of homecage control, respectively; $U = 41, p = 0.027$), but no difference in dorsal CA1 (similar = 282.8%; dissimilar = 472.4%; $U = 63, p = 0.29$) or S1 (similar = 848.3%; dissimilar = 995%; $U = 80, p = 0.86$).

Data are plotted as mean number of cells in each ROI for each animal, expressed as a percentage of homecage control group ± s.e.m.
Figure 6. Post-Training\textsubscript{1} infusion of pepR845A into ACC prevents AP5-insensitive learning during Training\textsubscript{2}.

(left) Two-way ANOVA revealed no main effect of peptide (pepR and SCR; $F_{1,23} = 0.064$, $p = 0.80$) or to-be-infused drug (AP5 and VEH; $F_{1,23} = 0.012$, $p = 0.91$), and no interaction of peptide and drug ($F_{1,23} = 0.439$, $p = 0.51$).

(right) Levene’s test revealed unequal variances across groups for LTM\textsubscript{2} freezing scores ($F = 4.82$, $p = 0.008$). Pairwise Mann-Whitney comparisons with Bonferroni correction for multiple comparisons (alpha = 0.025) revealed that SCR+VEH (median = 78.08%) and SCR+AP5 (median = 80.63%) groups froze comparably ($U = 17$, $p > 0.99$), whereas rats receiving pepR+AP5 froze significantly less (median = 60.21%) than those receiving pepR+VEH (median = 85.62%; $U = 7$, $p = 0.014$). n/group: SCR+VEH=5; SCR+AP5=7; pepR+VEH=7; pepR+AP5=8.

Data are expressed as mean percent of time spent freezing during LTM test ± s.e.m.
## STAR METHODS

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibodies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit anti-cFos polyclonal IgG</td>
<td>Santa Cruz Biotech</td>
<td>Cat#SC-52; RRID: AB_2106783</td>
</tr>
<tr>
<td>Biotinylated goat anti-rabbit IgG</td>
<td>Vector Labs</td>
<td>Cat#BA-1000; RRID: AB_2313606</td>
</tr>
<tr>
<td>Vectastain ELITE ABC Kit</td>
<td>Vector Labs</td>
<td>Cat#PK-6100; RRID: AB_2336819</td>
</tr>
<tr>
<td>DAB Peroxidase (HRP) Substrate Kit</td>
<td>Vector Labs</td>
<td>Cat#PK-4100</td>
</tr>
<tr>
<td><strong>Chemicals, Peptides, and Recombinant Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D,L-2-amino-5-phosphonopentanoic acid</td>
<td>Sigma-Aldrich</td>
<td>Cat#A5282-25MG</td>
</tr>
<tr>
<td>pepR845A</td>
<td>Anaspec Inc.</td>
<td>Custom peptide: TAT(47–57)KAMKVAKNPQ853</td>
</tr>
<tr>
<td>Scrambled pepR845A</td>
<td>Anaspec Inc.</td>
<td>Custom peptide: TAT(47–57)–VAKKNMACKP</td>
</tr>
<tr>
<td>Normal goat serum</td>
<td>Vector Labs</td>
<td>Cat#S-1000; RRID: AB_2336615</td>
</tr>
<tr>
<td><strong>Deposited Data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw and analyzed data</td>
<td>This paper; Mendeley data</td>
<td><a href="https://data.mendeley.com/datasets/nd2khkv3mr/draft?a=ef8cddd6135485b92673e49e2db46d">https://data.mendeley.com/datasets/nd2khkv3mr/draft?a=ef8cddd6135485b92673e49e2db46d</a></td>
</tr>
<tr>
<td><strong>Experimental Models: Organisms/Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat: Sprague-Dawley</td>
<td>Charles River</td>
<td>Nomenclature: Crl:SD Strain Code: 400</td>
</tr>
<tr>
<td><strong>Software and Algorithms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freezeframe Version 4</td>
<td>Coulbourn Instruments</td>
<td><a href="http://www.coulbourn.com/category_s/277.htm">www.coulbourn.com/category_s/277.htm</a></td>
</tr>
<tr>
<td>FIJI (ImageJ)</td>
<td>NIH</td>
<td><a href="https://fiji.sc">https://fiji.sc</a></td>
</tr>
<tr>
<td>SPSS Version 25.0</td>
<td>IBM</td>
<td><a href="http://www.ibm.com/analytics/spss-statistics-software">www.ibm.com/analytics/spss-statistics-software</a></td>
</tr>
<tr>
<td>PRISM Version 7</td>
<td>GraphPad</td>
<td><a href="http://www.graphpad.com/scientific-software/prism/">www.graphpad.com/scientific-software/prism/</a></td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10uL Glass Microinfusion Syringes</td>
<td>Hamilton</td>
<td>Ref#80300</td>
</tr>
<tr>
<td>Stereotoxic surgical apparatus</td>
<td>Kopf Instruments</td>
<td>Model #902</td>
</tr>
<tr>
<td>Microinfusion pump</td>
<td>K.D. Scientific</td>
<td>Cat#780200</td>
</tr>
<tr>
<td>Polyethylene infusion tubing</td>
<td>Intramedic</td>
<td>Cat#427406</td>
</tr>
<tr>
<td>Internal cannula</td>
<td>PlasticsOne</td>
<td>Model C317FD/SPC</td>
</tr>
<tr>
<td>Bilateral internal cannula</td>
<td>PlasticsOne</td>
<td>Model C235I/SPC</td>
</tr>
<tr>
<td>22-gauge external cannula</td>
<td>PlasticsOne</td>
<td>Model C313G/SPC</td>
</tr>
<tr>
<td>28-gauge external bilateral cannula</td>
<td>PlasticsOne</td>
<td>Model C235G-1.4/SPC</td>
</tr>
<tr>
<td>Obturator</td>
<td>PlasticsOne</td>
<td>Model C313D/1/SPC</td>
</tr>
<tr>
<td>Bilateral obturator</td>
<td>PlasticsOne</td>
<td>Model C235DC/SPC</td>
</tr>
<tr>
<td>Dust cap (bilateral cannula)</td>
<td>PlasticsOne</td>
<td>Model 303DC/1A</td>
</tr>
</tbody>
</table>

### Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Karim Nader (karim.nader@mcgill.ca).

### Experimental Model and Subject Details
Rats

Adult male Sprague-Dawley rats bred at Charles River Laboratories (Quebec, Canada) were used throughout these experiments. Rats were experimentally naïve and were housed individually in Nalgene cages in a temperature-controlled environment (21-23°C) with food and water provided ad libitum. Each rat was handled for at least 3 days before stereotaxic surgery. Each rat weighed 325-400g at the time of surgery (approximately 9-12 weeks old). Animals were maintained on a 12 hr light-dark cycle (07:00-19:00 hours light phase), and all experiments were conducted during the light phase. All procedures followed protocols approved by the McGill University Animal Care and Use Committee and were in accordance with the Canadian Council on Animal Care guidelines.

Method Details

Surgical Procedures.

Animals were anesthetized with a 1mL/kg IP injection of ketamine HCl (55.55mg/mL), xylazine (3.33 mg/mL), and domitor (0.27 mg/mL) drug cocktail. For analgesia during surgery and recovery, buprenorphine (0.324 mg/mL) or carprofen (5mg/mL) was administered subcutaneously at 1mL/kg. Each animal was mounted on a stereotaxic frame (Kopf Instruments), and stainless steel cannulae (22 or 28 gauge, Plastics One, Roanoke, VA) were bilaterally implanted in the brain targeting the regions of interest based on Paxinos and Watson’s atlas of the rat brain [78]. Coordinates for dHC cannulation were: A/P -3.6mm, L ±3.1mm, D/V -2.6mm (measured from bregma), ±10° from sagittal plane; for dHC+ACC cannulation the dHC coordinates were as above, and ACC were: A/P +2.6mm, L ±0.7mm, D/V -1.6mm (measured from dura), 0° from sagittal; and for dHC+vHC cannulation the dHC
coordinates were: A/P -3.7mm, L ±2.25mm, D/V -2.6mm, and vHC were: A/P -6.3mm, L ±5mm, D/V -6.0mm, both 0° from sagittal. These were stabilized with two layers of dental cement anchored to three jewelry screws drilled into the skull. Each rat was revived with a 0.67mL/kg IP injection of antisedan (5 mg/mL). Obturators (PlasticsOne) were inserted into each cannula to ensure patency. Surgeries were performed 7-10 days prior to the start of behavioral training, except for the experiment in Fig. 4 when surgeries were performed 7-9 days before Training2.

**Behavioral Apparatus.**

Two distinct training contexts were used in this study. In order to reduce generalization between the contexts, different visual, auditory, olfactory, and textural cues were used in each, and distinct routes were taken when transporting animals from the colony.

Context1 consisted of four Coulbourn (Whitehall, PA) conditioning boxes (30cm*26cm*33cm). All four side walls were made of transparent Plexiglas. Constant illumination was produced by a single light bulb located at the upper-middle of the right side wall of each chamber. The floor was composed of parallel stainless steel bars (radius=0.25 cm, 1 cm apart and 0 degrees horizontal inclination), connected to an animal shocker unit. The intensity of electric footshock was at 1 mA for 1s. Diluted vanilla scent was applied immediately prior to each training session. A digital camera was installed in front of the box for image recording and storage via Freezeframe software (Coulbourn). The experimental room remained brightly lit at all times.

Context2 consisted of Med-Associates (St. Albans, VT) fear conditioning boxes (29cm*25cm*25cm). The side walls of each chamber were made of aluminum panels. Two
lights were mounted on the right wall and an additional light was mounted on the left wall. The lights alternatingly flashed at a rate of 1 Hz. A plastic sheet was inserted to create a curved back wall. Black-and-white striped wallpaper was attached to the front wall (1 inch wide/each). The grid floor was similar to Context1 except each bar was narrower (radius=0.1 cm), had shorter inter-bar spacing (0.5 cm), and was tilted at a 7° plane. Wood-chip bedding was used to fill the floor tray such that it reached the surface of the grid floor. The intensity of electric footshock was set at 1.2 mA for 1 second. Peppermint scent was sprayed before each animal was put in the box. A fan provided ambient sound. A digital camera was mounted on the ceiling and videos were recorded for later analysis. The experimental room remained dimly lit at all times.

Previous studies revealed that whether Context1 or Context2 serves as the first training environment did not change the overall result - that is, the insensitivity of second learning to NMDAR blockade [7]. Thus, Context2 was used during Training2 throughout this study.

**Behavioral procedures**

*General behavioral protocol:* In all experiments each rat received two training tasks. In Figs. 1-3 we manipulated the learning content of Training1 and/or Training2. In Figs. 3, 4, and 6, we aimed to manipulate the memory trace for Training1 during the interval between Training1 and Training2. In all experiments animals were given Training1 and Training2 five days apart, except in Fig. 3 when this was extended to 31 days. Training1 was administered in Context1 (except several groups presented in Fig. S1 and S3), whereas Training2 always occurred in Context2. In all experiments AP5 or VEH was infused into dHC immediately before Training2, except in Fig. 2 when the infusion was given prior to the immediate shock phase of CPFE during Training2. In Figs. 4 and 6, pepR845A or Scrambled-pepR845A infusions were
given 24h after Training\textsubscript{1}. Unless otherwise noted, each training session was followed 24h later by a 4-min long-term memory (LTM) test in the same context.

*Delayed contextual fear conditioning (DC).* Unless otherwise noted, the one-day DC task consisted of a 3 min context exposure followed by 2 footshocks (30s inter-shock interval). In Fig. S4, the DC procedure was altered such that the pre-shock interval was 12 min, followed by 2 footshocks with a 1s inter-shock interval. In Fig. S5, 1 or 3 delayed shocks were administered. The rats were removed from the context 60s after the final footshock.

*Immediate shock.* In Figs. 1C, S2, and S3, Training\textsubscript{1} consisted of the immediate shock procedure. Each rat was placed into the context and rapidly received 1, 2, or 3 footshocks with a 1s inter-shock interval (see Results for specific experimental designs). The animal was then quickly (<10s) removed from the context. In Fig. 1D, 2, and 5, the immediate shock phase of two-phase conditioning differed slightly from this procedure (see below).

*Context exposure.* In some protocols Training\textsubscript{1} consisted of context exposure. This was either 4.5min in Context\textsubscript{1} (Fig. 1B) or Context\textsubscript{2}, or two 30 min sessions in Context\textsubscript{2} (Fig. S1). In Figs. 1D-3, the context exposure phase consisted of a 12min exploration session (as described in the CFPE section below).

*Context pre-exposure facilitation effect (CPFE).* In the two-day CPFE task, each animal was first pre-exposed to a conditioning chamber, and the next day was given 2 immediate shocks in the same context. During pilot testing we optimized the procedure such that only animals pre-exposed to the training context would show a reliable conditioned fear response (data not shown). This required both the context pre-exposure and immediate shock phases to differ slightly from those described above. During the context pre-exposure phase, each
animal remained in its homecage as it was transported to the context on a cart. Animals were given several minutes to acclimatize before being placed into the conditioning chamber for a 720s exposure session. Each rat was returned to the colony shortly after it was removed from the chamber. The next day animals were transported back to the same context for the immediate shock session. When CPFE was administered in Context$_1$, each rat was hand-carried to and from the context in a clear plastic mouse cage wrapped in an opaque white sheet. When conditioning was administered in Context$_2$, animals were transported to the context in an opaque metal bucket containing bedding. This was done so that animals could not merely rely on transportation cues to facilitate conditioning [18]. During the immediate shock session, each animal was removed from the transportation vessel and placed into the appropriate conditioning chamber. Fifteen seconds later the rat was given two 1s, 1.35mA footshocks, with a 1s inter-shock interval. The rat was removed several seconds after the last shock, placed into the transportation vessel, and returned to the colony. This higher footshock intensity was used because it was found to evoke freezing comparable to that elicited by the DC procedure during pilot experiments. The LTM test following CPFE occurred as usual, with rats transported in their home cages on a cart, ensuring that freezing was elicited by the context and not by transportation cues.

**Behavioral measurement.** Memory was assessed as the percentage of time that the animal exhibited freezing behavior, defined as total immobilization except for movements required for respiration [25, 27]. An observer blinded to treatment condition measured freezing time. Each 4-minute LTM test was divided into 30-second intervals, and the results are presented as the percentage of freezing time averaged across all 8 intervals. Pre-shock freezing during Training$_2$ was also assessed as the percentage of the 30s interval from 150s to
180s after initial placement into the context.

**Group assignment**

In all experiments animals were randomly assigned to each behavioral training condition, and in Figs. 4 and 6 animals were randomly assigned to each peptide group (pepR/Scr). In all experiments animals were assigned to receive Training$_2$ drug treatments (VEH or APS) by matching pairs of rats that froze comparably during LTM$_1$ test, then randomly splitting these pairs between the conditions. Group assignment and drug administration were each performed by experimenters blinded to the behavioral protocol. Sample size estimates were determined based on effect sizes observed in previous reports using similar behavioral assays [6, 7, 9].

**Drug delivery**

All drugs were administered via 28- or 33-gauge stainless steel injectors (for hippocampus and ACC, respectively) extending +0.5mm from the tip of each external cannula, attached by polyethylene tubing (Intramedic #427406) to 10uL Hamilton syringes driven at 0.4 μL/min by a K.D. Scientific microinfusion pump. NMDAR antagonist D,L-2-amino-5-phosphonopentanoic acid (AP5; Sigma; 5μg/2μl/hemisphere) was infused at a rate of 0.4μl/min. An equivalent volume of physiological saline served as vehicle control (VEH). PepR845A (TAT(47–57)-KAMKVAKNPQ$_{853}$) and scrambled Scr-pepR845A (TAT(47–57)-VAKKNMAKQP; Anaspec Inc.) were each dissolved in artificial cerebrospinal fluid (aCSF, 150mM NaCl, 3mM KCl, 1.4mM CaCl$_2$, 0.8mM MgCl$_2$, 0.8mM Na$_2$HPO$_4$, and 0.2mM NaH$_2$PO$_4$, pH 7.4) to a concentration of 30μM and infused at 2μl/hemisphere into dHC, 1.25μl/cannula into d+vHC, and 0.5μL/hemisphere into ACC. The pH-value of each solution was adjusted to
Histology

At the end of every experiment, each brain was removed and post-fixed in 10% formalin-saline, 20% sucrose solution (for cryo-protection to preserve the morphology). They were cryo-sectioned at 50μm thickness. The slides were examined by bright-field light microscopy (Olympus Corporation, Japan, model IX81) for cannula placements by an experimenter blind to the group assignments. Only animals with injector tips bilaterally positioned within the dHC, dHC+vHC, or dHC+ACC were included in the data analysis. Rats with extensive hippocampal and/or cortical damage were excluded from analysis.

Exclusion criteria

In addition to rats removed due to technical issues (i.e. cannula blockage, incorrect cannula placement, apparatus malfunction), there were also several predefined behavioral exclusion criteria. These exclusions are listed by experiment in Table S1. A small minority of rats in Figs. 1A 1D, 2, and 4 exhibited <10% freezing during LTM1, and were therefore deemed not to have acquired Training1. Moreover, rats in these experiments that froze for more than 35% of the pre-shock interval during Training2 were deemed to already possess a robust freezing response to this novel context. These exclusion criteria were included to ensure that each rat exhibited clear evidence of learning during both Training1 and Training2. The small subset of rats that generalized between training contexts already exhibited a fear response to Context2 prior to Training2, thus without exclusion could not be readily distinguished from animals who were simply unaffected by the AP5/VEH infusion. These exclusion criteria were not included for Figs. 3, 4, and 6 because we predicted that the experimental treatments could alter Training1 memory retention, which would potentially also impact fear generalization.
Finally, a combined total of 6 rats were excluded from Figs. 1C, S2, and S3 because they exhibited a strong (>35%) fear response during LTM following the Training immediate shock procedure. The aim of this experiment was to expose animals to shock during Training without evoking a conditioned fear response, thus animals reliably freezing during LTM were considered to have formed a robust context-shock association.

**Immunohistochemistry**

Each animal was deeply anesthetized 90 minutes after Training with the same ketamine cocktail used during surgery. The animal was then perfused transcardially with saline and 4% paraformaldehyde (in 0.1 M PB; 4°C), the brain was extracted and submerged in 4% paraformaldehyde for 4h then 20% sucrose solution for 48h (both at 4°C), before being rapidly frozen in 2-methylbutane chilled on dry ice for storage at -80°C. Each brain was then sliced on a frozen microtome at a thickness of 40μm and stored in antifreeze at -20°C. Three slices (approximately 40μm apart) from each region of interest (ROI) were then stained for c-Fos protein. Briefly, the floating slices for each ROI from each animal were washed in PBS then 0.3% hydrogen peroxide, then incubated in 2% BSA, 2% NGS blocking solution (Vector Labs #S-1000) for 60min. The slices were then incubated in rabbit anti-cFos polyclonal IgG (Santa Cruz Biotech #SC-52, diluted 1:1000 in blocking solution) overnight (16h) at 4°C. They were then washed repeatedly in PBS before applying secondary antibody (biotinylated goat anti-rabbit IgG; Vector Labs, Birmingham CA #BA-1000, diluted 1:500 in blocking solution) for 60min. Slices were again washed and incubated for 60min in Vectastain ELITE ABC reagent (Vector Labs #PK-6100). After washing, DAB (Vector Labs #SK-4100) was then applied for 90s and promptly rinsed in PBS. The slices were slide-mounted, dehydrated, and cover-slipped.
Images were captured on an Olympus bright-field light microscope (model IX81) at both 4x and 10x objective magnifications at a resolution of 1392 x 1040 pixels.

Each slice was analyzed bilaterally for each ROI using a semi-automated counting procedure using NIH ImageJ software. Each representative image was captured via a 10x objective lens, manually cropped within the typical boundaries of each brain structure, and converted to a binary image based on a standardized threshold value. A watershed algorithm was applied to each image to distinguish partially overlapping cells, and then particles with a minimum size of 30 pixels$^2$ and circularity of 0.3 or greater were tallied. All semi-automated cell counts were visually inspected to identify miscounted particles. Approximately one quarter of slices were also quantified manually to confirm the validity and reliability of the automated procedure. An acceptable correlation was obtained between our manual and semi-automated counts of cFos positive neurons (Pearson’s $r = 0.88$, $p < 0.001$). The mean cell count for each ROI was then normalized to the homecage control group average to provide a percentage over the baseline number of cFos-positive neurons. One rat was excluded from analysis due to delays inducing deep anesthesia prior to perfusion. All slices from 7 brains exhibited clear evidence of poor fixation (including the total absence of detectable cFos-positive neurons), and the decision to exclude these animals was performed blind to group assignment. A small number of sections in which the ROI contained tissue damage or distorted mounting media were also excluded (blind to group), hence the mean cell counts for these animals was calculated from the remaining subset of slices.

**Quantification and Statistical Analysis**

All data analysis was performed by experimenters blinded to the group identity of each animal. Statistical analyses were performed using SPSS (IBM) or PRISM (GraphPad)
software. Sample size (n) corresponds to the number of rats per group, and is listed within each figure caption. Freezing scores were analyzed using two-tailed independent-samples t-tests (with Bonferroni correction for multiple comparisons) or two-way between-subjects ANOVAs. Significant 2 x 2 interactions were followed by posthoc pairwise comparisons with Tukey correction for multiple comparisons. Homogeneity of variance (Levene’s test) and normality (D’Agostino & Pearson omnibus test, or Shapiro-Wilk test for instances in which n < 8) were evaluated to ensure the assumptions of each statistical procedure were met. In the case of violation of these assumptions, Welch’s unequal variances t-tests or non-parametric Mann-Whitney (MW) U tests with Bonferroni correction were substituted where appropriate. Normalized cFos-positive cell counts were analyzed with planned MW comparisons between similar and dissimilar groups for each ROI.

Type-one error rate (α) was set at 0.05 for all comparisons, and corrected for multiple comparisons as described. Mean freezing for each group was reported as percent of the assessment interval ± the standard error of the mean (s.e.m.).

Data and Software Availability

The authors confirm that all raw or analyzed data supporting this study will be distributed promptly upon reasonable request. Behavioral freezing scores for each experiment have also been deposited onto Mendeley Data (doi:10.17632/nd2khkv3mr.1). No new software or code was generated in this study. All software used (SPSS, PRISM, ImageJ/FIJI, FreezeFrame) is commercially or freely available, as listed in the Key Resources Table.