Research

# Distributed learning episodes create a context fear memory outside the hippocampus that depends on perirhinal and anterior cingulate cortices

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**Damage to the hippocampus (HPC) typically causes retrograde amnesia for contextual fear conditioning. Repeating the conditioning over several sessions, however, can eliminate the retrograde amnesic effects. This form of reinstatement thus permits modifications to networks that can support context memory retrieval in the absence of the HPC. The present study aims to identify cortical regions that support the nonHPC context memory. Specifically, the contribution of the perirhinal cortex (PRH) and the anterior cingulate cortex (ACC) were examined because of their established importance to context memory. The findings show that context memories established through distributed reinstatement survive damage limited only to the HPC, PRH, or ACC. Combined lesions of the HPC and PRH, as well as the HPC and ACC, caused retrograde amnesia, suggesting that network modifications in the PRH and ACC enable context fear memories to become resistant to HPC damage.**

**[Supplemental material is available for this article.]**

Damage to the hippocampus (HPC) causes retrograde amnesia for certain kinds of memories (Scoville and Milner 1957; Rempel-Clower et al. 1996; Moscovitch et al. 2006). There are some experiments showing that these memories can become independent of the HPC, meaning that the memories no longer critically require the HPC for storage or retrieval (Kim and Fanselow 1992; Clark et al. 2002; Kirwan et al. 2008; Lehmann et al. 2009). One proposed process for how memories may become independent of the HPC is long-term systems consolidation. This view suggests that certain memories are strengthened in neocortical structures over a lengthy period of time and is supported by the findings of temporally graded retrograde amnesia, in which recently, but not remotely, acquired memories are lost after HPC damage (Marr 1971; Alvarez and Squire 1994; McClelland et al. 1995; Nadel and Moscovitch 1997; Frankland and Bontempi 2005; Sekeres et al. 2018). For instance, some patients cannot remember life episodes that occurred a short time before the onset of their HPC damage, but can remember episodes that occurred a decade or more before (Scoville and Milner 1957; Rempel-Clower et al. 1996; Kirwan et al. 2008).

The Distributed Reinstatement Theory (DRT) proposes another account for how memories may become resistant to HPC damage. The DRT postulates that reinstatements of the memory or repetition of the learning episode, in some form, strengthens the memory in nonHPC systems (Sutherland et al. 2010). With a sufficient number of reinstatements, the memory can come to be expressed without a necessary contribution from the HPC. Evidence for the DRT has come from our work examining the amnesic effects of HPC lesions in rats tested in contextual fear conditioning (Lehmann et al. 2009). This task involves the pairing of a context (a configuration of static cues) with an aversive stimulus (foot shock). Upon reintroduction into the context, the rats exhibit species-specific behaviors indicative of fear, including freezing (the absence of movement with the exception of breathing). We found that HPC damage after learning severely impairs retrieval of context fear established by 12 context–shock pairings in a single session, a pattern of results also obtained in numerous other studies (Wiltgen et al. 2006; Lehmann et al. 2007, 2013; Sutherland et al. 2008; Sparks et al. 2011, 2013; Broadbent and Clark 2013). Therefore, following a single contextual fear conditioning session, HPC damage causes severe retrograde amnesia. We also found, however, that distributing the 12 context–shock pairings over 11 sessions, across 6 d, establishes a memory that survives even very extensive HPC damage (Lehmann et al. 2009). Here we ask which cortical regions might be essential for the retention and expression of context memories for which the HPC is no longer necessary. The purpose of the current study is to partially answer this question by examining the role of the perirhinal cortex (PRH) and anterior cingulate cortex (ACC) in reinstated contextual fear conditioning. These structures were selected as primary candidates because damage or inactivation of the PRH or the ACC can impair context fear memory involving a single learning episode (Sacchetti et al. 1999; Bucci et al. 2000; Burwell et al. 2004; Frankland et al. 2004; Tang et al. 2005; Einarsson and Nader 2012; Einarsson et al. 2015). The role of these two structures in context fear conditioning is seen as idiosyncratic with the PRH playing a role in polymodal processing of the context (Bucci et al. 2000) and the ACC in context generalization (Einarsson et al. 2015). Of importance for this study, however, both structures are considered part of a context fear network.

We hypothesized that if the PRH and ACC are critical components of the nonHPC memory system supporting context fear established over multiple, distributed sessions, then damaging one of these structures in combination with the HPC should impair context fear memory. We argue that combined damage is

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necessary because some evidence suggests that the HPC does not completely disengage from supporting a memory (see Sutherland et al. 2019). To assess this hypothesis, we conducted the following three experiments: (1) We examined the effect of HPC lesions on contextual fear conditioning acquired in a single session, expecting retrograde amnesic effects; (2) we examined individual and combined lesions of the HPC and PRH following distributed sessions of contextual fear conditioning, with the expectation that only the combined lesions would cause retrograde amnesia; and (3) we examined individual and combined lesions of the HPC and ACC following reinstated contextual fear conditioning, again with the expectation that only the combined lesions would cause retrograde amnesia.

## Results

### Experiment 1—single-session context fear memory is disrupted by postlearning HPC damage

The goal of the current experiment was to confirm the retrograde amnesic effects of HPC damage on contextual fear memory acquired in a single conditioning session (10 context–shock pairing delivered across a 30-min session) (Supplemental Fig. S1). These conditioning parameters were selected because they match the number of context–shock pairings, as well as, extent of context exposure time used in the later experiments involving distributed/reinstated contextual fear conditioning.

As illustrated in Figure 1, the HPC group ($n = 12$) exhibited significantly less freezing than the Sham group ($n = 8$) during the retention test [$t_{(18)} = 3.71$, $P < 0.01$]. Thus, damaging the HPC 7–9 d after a single contextual fear conditioning session caused retrograde amnesia. This also implies that contextual fear conditioning acquired in a single session is normally dependent on the HPC. Importantly, the group difference on the retention test cannot be accounted for by learning differences. The freezing levels during the conditioning session between the Sham ($M = 68.7$, SEM = 4.5) and HPC ($M = 67.6$, SEM = 4.4) rats did not significantly differ [$t_{(18)} = 0.16$, $P = 0.88$], indicating that the groups were properly matched prior to surgery. In addition, the histological analyses revealed the lesions were specific to the HPC. A description of the lesions is in the Supplemental Material and lesion size descriptive statistics are in Table 1.

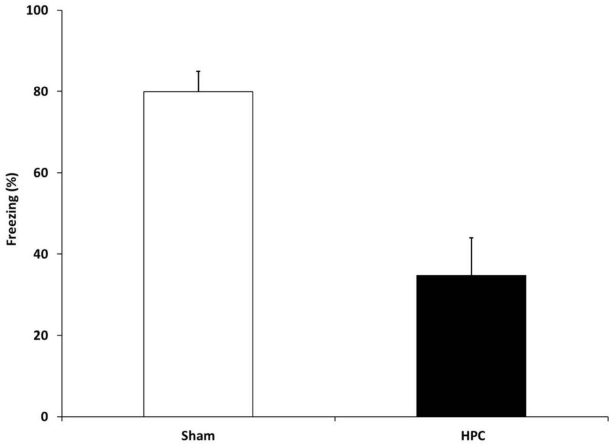


**Figure 1.** Mean (+SEM) percent time freezing during the retention test in experiment 1. The HPC rats froze significantly less than the Sham control group ($P < 0.05$), suggesting that HPC damage caused retrograde amnesia. Hence, context fear memory, acquired in a single session, was dependent on the HPC.

**Table 1. Descriptive statistics for the percent lesion size in each experiment.**

| | | Mean | SEM | Smallest | Largest |
|---|---|---|---|---|---|
| Experiment 1 | HPC | 77.1 | 3.3 | 57.3 | 90.3 |
| Experiment 2 | HPC | 77.4 | 2.2 | 53.5 | 92.6 |
| | PRH | 65.1 | 3.0 | 39.4 | 79.4 |
| Experiment 3 | HPC | 69.4 | 2.6 | 58.3 | 86.1 |
| | ACC | 63.7 | 1.9 | 53.5 | 76.7 |

### Experiment 2—reinstated context fear memory is intact after HPC damage, but not after combined HPC and PRH damage

The previous experiment demonstrated that contextual fear conditioning acquired in a single session is normally dependent on the HPC, yet other evidence suggests that reinstated or distributed fear conditioning becomes resistant to HPC damage (Lehmann et al. 2009; Lehmann and McNamara 2011). Thus, distributed reinstatements can alter the system supporting context fear memory by strengthening its representation within nonHPC structures. The present experiment aimed to (1) replicate this finding and (2) identify whether the PRH is part of this nonHPC memory system. The PRH was selected as a candidate structure because of its established role in context memory (Sacchetti et al. 1999; Bucci et al. 2000; Burwell et al. 2004). We hypothesized that damage to either structure alone would fail to cause amnesia because the memory would be sufficiently represented in the other system. However, we predicted that combined damage to the HPC and a significant portion of the nonHPC memory system (i.e., PRH) would cause amnesia for distributed contextual fear conditioning. To examine these two possibilities, the same rats were tested with damage to either the HPC or PRH independently and then with combined damage. Figure 2 illustrates the experimental design.

In total, the data from six rats were removed from the statistical analyses (see "Exclusion Summary" in the Supplemental Material). In addition, presurgery conditioning performance was comparable across groups [Sham ($M = 69.6$, SEM = 10.1, $n = 8$), HPC ($M = 58.4$, SEM = 8.5, $n = 7$), and PRH ($M = 64.0$, SEM = 8.1, $n = 9$; $F_{(2,21)} = 0.366$, $P = 0.70$]. Therefore, the behavioral performance of each group on the tests following surgery cannot be accounted for by differences in initial learning.

#### *Retrograde amnesia tests*

On the first retention test, as shown in Figure 3A, freezing did not significantly differ across groups [$F_{(2,21)} = 1.220$, $P = 0.32$], suggesting that lesions initially circumscribed to either the HPC or PRH failed to cause retrograde amnesia for distributed contextual fear conditioning. However, on the second retention test, given after the rats received the combined HPC and PRH lesions, the lesion group now showed significantly less freezing than the Sham control group [$t_{(22)} = 5.33$, $P < 0.001$]. These data are illustrated in Figure 3B. Thus, combined lesions of the HPC and PRH caused retrograde amnesia for distributed context fear memory.

#### *Reconditioning*

At the end of Retention 2, the rats were administered two additional shocks and given a third test a day later. This reconditioning aimed to assess whether the amnesic effect of the lesions was possibly due to a more general behavioral performance deficit. Although the freezing behavior of the Sham ($M = 82.3$, SEM = 9.0) and HPC + PRH ($M = 59.1$, SEM = 5.5) groups still significantly differed on this test [$t_{(22)} = 2.33$, $P < 0.05$], a paired sample *t*-test

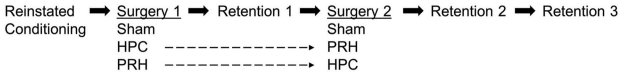


**Figure 2.** Illustration of the experimental design used in experiment 2. The reinstated conditioning involved the same number of context–shock pairings and amount of time spent in the conditioning chamber as in experiment 1. However, the conditioning took place over 10 sessions rather than a single session, similar to the protocol used by Lehmann et al. (2009). Specifically, the rats received two conditioning sessions per day for five consecutive days (Reinstated Conditioning). Each session lasted 3 min, during which the rats only received a single shock. Three days to 5 d after the Reinstated Conditioning, the rats either received Sham, HPC, or PRH damage (Surgery 1). Following the surgical recovery period, the rats were returned to the conditioning chamber for a 3-min retention test (Retention 1) to ensure that the memory had become resistant to damage of the HPC memory system or of the nonHPC system (i.e., PRH). Three days to 5 d later the same rats received a second surgery to damage the structure that had not previously been lesioned (Surgery 2), resulting in two groups: Sham and HPC + PRH. Again, following the surgical recovery period, the rats were tested for retention (Retention 2), which involved a 5-min retention test followed by a 2-min reconditioning session with two shocks. The following day, the rats were given an ultimate 5-min retention test (Retention 3) to assess memory of the reconditioning.

revealed a significant increase in freezing from Retention 2 to Retention 3 [$t_{(15)} = -6.64$, $P < 0.001$] for the HPC + PRH group. Thus, the Retention 3 findings show that the rats with HPC + PRH damage were capable of freezing and that the amnesia on Retention 2 was not a mere performance deficit.

### *Relationship between damage extent and retention performance*

We also examined whether the retention performance impairment was associated with the extent of the lesions as reported in other studies (Lehmann et al. 2007; Sutherland et al. 2008; Scott et al. 2016). Supplemental Figure S2 shows representative damage in a rat that received combined HPC + PRH damage and Table 1 includes the descriptive statistics for the extent of the lesions. Supplemental Figure S4 illustrates the correlation between freezing percent during Retention 2 and total HPC + PRH damage extent. Following the removal of an outlier (exceptionally high levels of freezing) (red triangle in Supplemental Fig. S4), it was found that damage extent and freezing were not significantly correlated [$r_{(15)} = -0.202$, $P = 0.47$].

## Experiment 3—reinstated context fear memory is also impaired after combined HPC and ACC damage

This experiment aimed to examine whether the ACC, which plays a known role in contextual fear conditioning (Frankland et al. 2004; Tang et al. 2005; Einarsson and Nader 2012; Einarsson et al. 2015), is also a contributing structure within the nonHPC memory system for reinstated context fear. This experiment involved the same experimental design as in experiment 2 (see Fig. 2) with the exception that the ACC was damaged rather than the PRH. Again, it was predicted that only combined damage to the HPC and ACC would cause retrograde amnesia.

The data from six rats were excluded from the statistical analyses (see "Exclusion Summary" in the Supplemental Material). Again, conditioning performance across groups was properly equated prior to surgery [Sham ($M = 77.3$, SEM = 4.3, $n = 4$), HPC ($M = 70.6$, SEM = 12.9, $n = 6$) and ACC ($M = 67.0$, SEM = 10.3, $n = 7$); $F_{(2,15)} = 0.189$, $P = .83$].

### *Retrograde amnesia tests*

On the first retention test, as shown in Figure 4A, freezing did not significantly differ across groups [$F_{(2,14)} = 0.504$, $P = 0.62$], suggesting that neither the circumscribed HPC or ACC lesions caused retrograde amnesia for reinstated contextual fear conditioning. Similarly, freezing on the second retention test (Fig. 4B), given once the rats had the combined HPC and ACC lesions, did not significantly differ between the two groups [$t_{(15)} = 0.798$, $P = 0.44$]. Thus, the combined lesions seemed insufficient to cause retrograde amnesia. Because memory was not impaired on the second test, reconditioning test data (Retention 3) were inconsequential [Sham ($M = 79.1$, SEM = 12.1) and HPC + ACC ($M = 72.2$, SEM = 7.6); $t_{(15)} = 0.449$, $P = 0.66$].

### *Relationship between damage extent and retention performance*

Figure 4C depicts the relationship between the total HPC + ACC volume damage and Retention 2 freezing. A significant negative Pearson correlation was found [r(13) = −0.684, $P < 0.01$], indicating that freezing decreased as lesion size increased. Partial correlations were also conducted to assess the independent relationship of each structure with performance on Retention 2. When controlling for the extent of the ACC lesions, the correlation between the HPC lesion size and freezing was not significant ($r_{(10)} = -0.126$, $P = 0.70$). In contrast, when controlling for the extent of the HPC lesions,

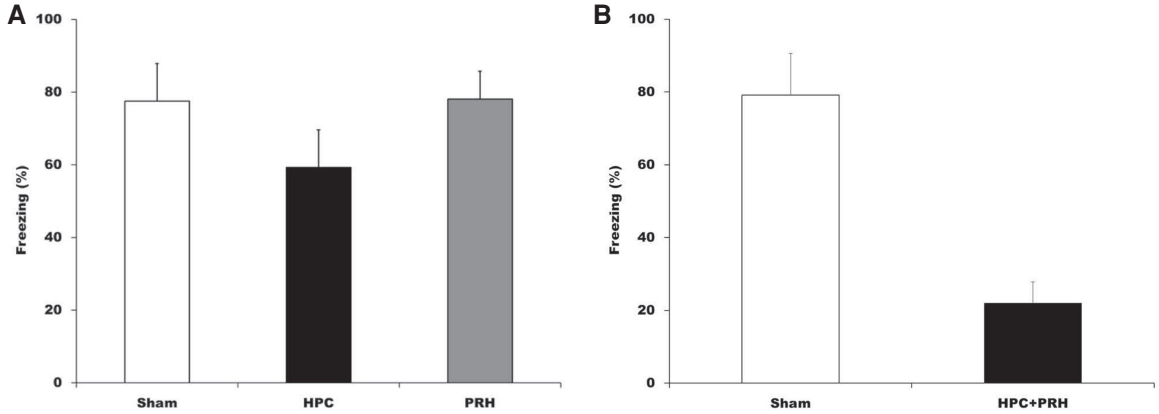


**Figure 3.** Mean (+SEM) percent time freezing during the first two retention tests in experiment 2. (*A*) During Retention 1, the freezing levels of the Sham, HPC, and PRH groups did not significantly differ ($P > 0.05$), suggesting that damage to either the HPC or PRH alone failed to cause retrograde amnesia. (*B*) During Retention 2, however, the HPC + PRH group froze significantly less than the Sham group ($P < 0.001$), suggesting that the combined damage caused retrograde amnesia. Therefore, the HPC and PRH conjointly support reinstated context fear memory.

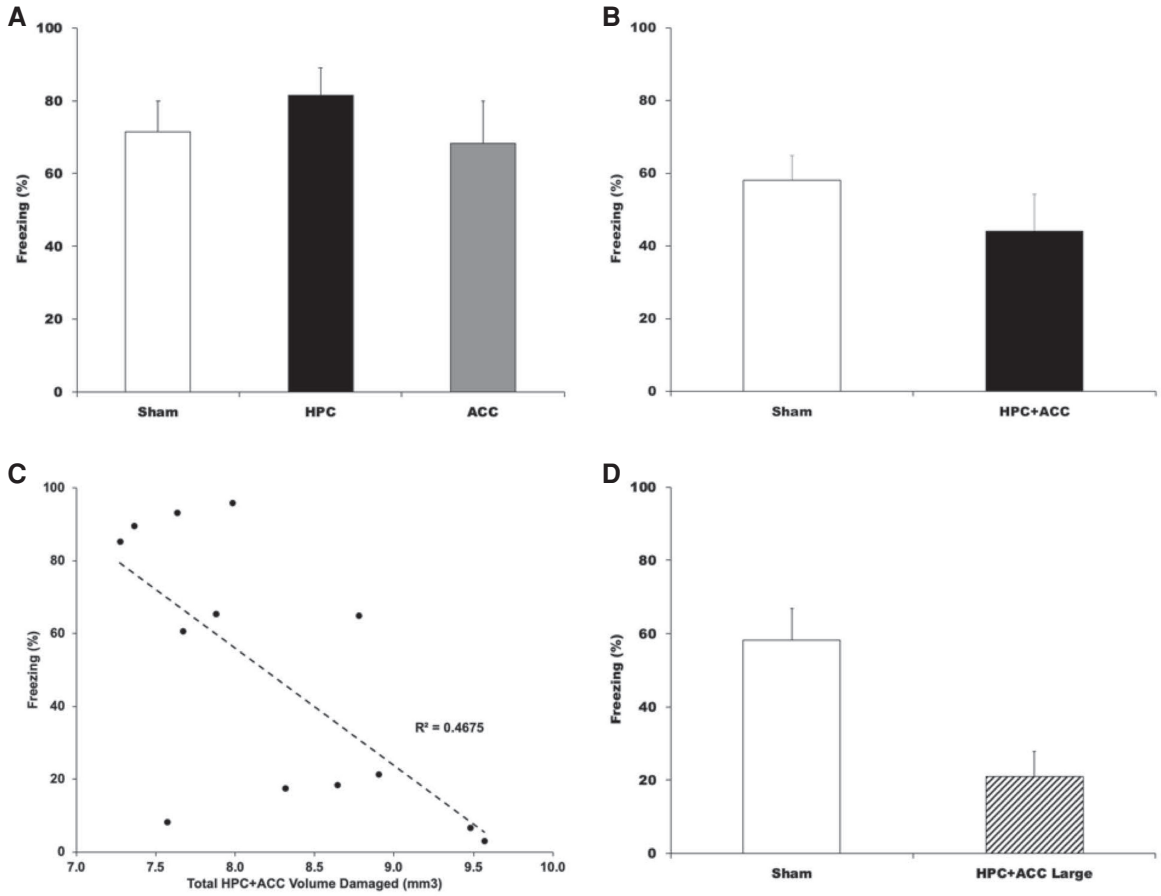


**Figure 4.** (*A*) During Retention 1, the freezing levels (mean + SEM) of the Sham, HPC, and ACC groups did not significantly differ ($P > 0.05$), suggesting that damage to either the HPC or ACC alone failed to cause retrograde amnesia. (*B*) During Retention 2, the freezing behavior did not significantly differ ($P > 0.05$) between Sham and the HPC + ACC rats. (*C*) Scatter plot and regression line showing that freezing behavior during the Retention 2 significantly decreased ($P < 0.05$) with increasing HPC + ACC damage. A partial correlation also showed that freezing and lesion size relationship was accounted for by the increase in ACC lesion size ($P < 0.001$). (*D*) Freezing performance on Retention 2 of the six rats with the largest amount of ACC damage (>64% damage). This subset of HPC + ACC rats with the most ACC damage froze significantly less than the control group ($P < 0.05$) and suggests that reinstated context fear memory is impaired following HPC damage and extensive ACC lesions.

the correlation between the ACC lesion size and freezing remained significant ($r_{(10)} = -0.682$, $P < 0.05$), suggesting that retrograde amnesia emerges following the combined lesions if the ACC damage is sufficiently extensive.

The significant relationship between the extent of the ACC lesion size and retention performance lead to a reanalysis of the data on the second and third retention tests. Specifically, the six HPC + ACC rats with the greatest ACC lesions (64.5%–76.7% damage) (see Supplemental Fig. S3 for a representative example) were compared with the Sham control group (see Fig. 4D). On the second retention test, conducted after the rats received the combined HPC and ACC lesions, the HPC + ACC Large group now showed significantly less freezing than the Sham group [$t_{(8)} = 3.34$, $P < 0.01$]. Thus, combined lesions of the HPC and ACC caused retrograde amnesia for reinstated context fear memory.

Even when limited to the six rats with the most ACC damage, the combined HPC and ACC damage did not cause an overall performance deficit. On Retention 3, which assessed freezing after reconditioning, the HPC + ACC Large lesion subgroup did not freeze significantly less than the control group [HPC + ACC Large ($M = 58.4$, SEM = 10.5); $t_{(8)} = 1.271$, $P = 0.24$]. In addition, this HPC + ACC Large subgroup showed a significant increase in freezing from Retention 2 to Retention 3 [$t_{(5)} = -7.728$, $P < 0.001$].

## Discussion

The main goal of the present study was to examine whether the PRH and ACC are part of the network supporting reinstated context fear memory that has become resistant to HPC damage. In experiment 1, HPC damage following a single contextual fear conditioning session caused retrograde amnesia, implying that the context fear memory learned in a massed session is dependent on the HPC. In experiments 2 and 3 the findings clearly demonstrated that distributing the same conditioning (number of context–shock pairings) over several sessions made the memory become resistant to extensive HPC damage. Furthermore, these two experiments demonstrated that damage to the PRH or ACC alone was also insufficient to produce amnesia. However, the combined damage to the HPC and PRH as well as to the HPC and ACC, if extensive enough, resulted in retrograde amnesia for the reinstated context fear memory, with both lesion groups freezing less than their sham counterparts. Moreover, these deficits are not simply the result of a general performance deficit because the rats with lesions were able to show significant freezing behavior following postsurgery reacquisition. Thus, the PRH and ACC are part of the network supporting a context fear memory that become resistant to HPC damage.

The current findings support the hypothesis put forward by the DRT, which states that reinstatements or repetitions of a learning episode strengthen the memory representation in nonHPC memory structures (Sutherland et al. 2010). Specifically, we found that complete, or almost complete, lesions of the HPC alone failed to produce retrograde amnesia for reinstated context fear memory. This result replicates our prior reinstatement findings (Lehmann et al. 2009; Lehmann and McNamara 2011). This is in contrast to the amnesic effects observed in experiment 1 of this study, as well as those from many other studies showing that lesions of the HPC cause retrograde amnesia for context fear acquired in a single session (Wiltgen et al. 2006; Lehmann et al. 2007, 2013; Sutherland et al. 2008; Sparks et al. 2011, 2013; Broadbent and Clark 2013). Moreover, because the rats in our reinstatement and single session conditions were matched for context exposure, context–shock pairings, as well as the interval between initial learning and surgery, the differing amnesic outcomes following the lesions are most parsimoniously explained by the distributed reinstatements creating a strong enough representation of the memory in other networks to withstand damage restricted to the HPC.

This study was not designed to examine theories of systems consolidation, but our observed modification of nonHPC cortical networks over distributed reinstatements may have important implications for these views, whether (1) the Standard Model of Consolidation or (2) the Multiple Trace Theory and its more contemporary version, the Trace Transformation Theory. The Standard Model of Consolidation (Squire 1992; Alvarez and Squire 1994; Squire and Alvarez 1995; Frankland and Bontempi 2005) suggests that the HPC is temporarily involved in indexing or binding the detailed-rich representations or traces in the neocortical network. Because of processes, such as replay during rest periods and reactivations of the memory, that would occur over weeks, months, and years, the neocortical trace would gradually strengthen with time. Eventually, the detailed-rich representation would become truly independent of the HPC, meaning that the HPC would no longer contribute to the retrieval process of the memory in the neocortical network. In contrast, the Multiple Trace Theory as well as the Trace Transformation Theory (Nadel and Moscovitch 1997; Winocur et al. 2010; Sekeres et al. 2018) postulate that the HPC has a continued role in the detailed-rich memory of the mnemonic episode. In addition, processes again such as replay and reactivations, over a protracted period, would enable other cortical structures to extract and establish a new schematic/gist-like/generalized memory trace. Importantly, in these transformation theories, the detailed-rich representation continues to persist in the HPC, leading to the consolidation of a modified representation in other cortical networks.

First and of importance for both of the aforementioned consolidation theories, the distributed reinstatements strengthened the memory in nonHPC neural networks over a very brief period, one that is much shorter than those of other studies assessing the role of the HPC in context fear memory (Kim and Fanselow 1992; Maren et al. 1997; Anagnostaras et al. 1999; Quinn et al. 2008; Winocur et al. 2013). Furthermore, in the current study, despite an equal opportunity for systems interaction in our massed (experiment 1) and distributed experiments (experiments 2 and 3—Retention 1), the HPC lesion amnesic effects were only mitigated by the distributed reinstatements. It is possible that the repeated conditioning sessions caused more bouts of systems interaction, resulting in accelerated systems consolidation, but instances of accelerated systems consolidation require an existing and well-established schematic representation (see Tse et al. 2007, 2011). Within our study, there was no pre-existing context fear schema because the rats were initially naïve to the context and shock experience. Nevertheless, the straightforward distributed conditioning approach in our study reliably altered the trace in nonHPC networks.

Second, we only observed clear retrograde amnesic effects after distributed conditioning if either the PRH or ACC were damaged together with the HPC. This outcome is only possible with continued contribution from the HPC. This is consistent with Multiple Trace Theory and Trace Transformation Theory, but not the Standard Model of Consolidation with the latter suggesting that the HPC disengages once the memory has been consolidated in the neocortex. Our findings also corroborate imaging work, immediate early gene expression studies, and optogenetic silencing and activation experiments demonstrating a persistent involvement of the HPC in long-term memory (Teixeira et al. 2006; Goshen et al. 2011; Barry et al. 2016; Carr et al. 2016; Kitamura et al. 2017; Vetere et al. 2017; Bonnici and Maguire 2018). Hence, these combined observations score against theories that posit that the HPC becomes disengaged as modification of cortical networks proceed.

Third, perhaps our distributed conditioning episodes created a schematic representation outside the HPC, a possibility that would seemingly support Trace Transformation Theory. According to the transformation model on context fear conditioning, systems consolidation processes would enable the development of context fear gist-like memory in cortical areas, such as the medial prefrontal cortex and the ACC, by extracting general features from the detailed-rich context memory that is dependent on the HPC. Supporting this view, Einarsson et al. (2015) found that inactivation of the HPC impairs context fear discrimination, which require retrieving detailed-rich information. In contrast, they also found that inactivation of the ACC impaired context fear generalization, meaning the gist-like representation of the conditioning. This double dissociation could account for our findings in experiment 3. Specifically, damaging the HPC alone after the distributed conditioning could have impaired the detailed-rich HPC-dependent representation and left an intact generalized representation that developed in the ACC, one sufficient to support retention performance. Conversely, damaging the ACC alone could have impaired the gist-like representation and left an intact and now strong detailed-rich memory in the HPC, again sufficient for successful retrieval on the retention test. Accordingly, combined damage to the HPC and ACC should cause loss of both types of representation and results in retrograde amnesia, which we found. This transformation account, however, is not supported by the Lehmann et al. (2009) findings showing that rats with HPC damage are successful at context discrimination following distributed fear conditioning, albeit very different contexts. Also, it cannot explain the findings from experiment 2 involving combined HPC and PRH lesions. The PRH, contrary to the ACC, is not involved in processing commonalities or generalization, but is critical for differentiation of stimuli with overlapping properties (Kivisaari et al. 2012; Clarke and Tyler 2014). Hence, after combined damage to the HPC and PRH, an ACC-dependent semantic representation should have been able to support retention performance but did not. There is evidence that the ventral HPC plays a role in generalized fear conditioning in conjunction with the ACC (Cullen et al. 2015; Ortiz et al. 2019), raising the possibility that damaging the ventral HPC could have impaired enough of the semantic representation to account for the retrograde amnesic effects seen in the HPC + PRH rats. This explanation, however, would be surprising given that extensive HPC and ACC damage was needed to disrupt the discussed semantic representation in experiment 3.

The benefits of distributed over massed learning were reported as early as 1885 by Ebbinghaus (see Ebbinghaus 1964) and it has been widely reproduced across a variety of memory tasks and species (Fanselow et al. 1993; Menzel et al. 2001; Bello-Medina et al. 2013; Kwon et al. 2015). The cognitive explanations for the

changes are extensive and there is clear evidence that distributed learning promotes more cellular consolidation and reconsolidation processes than massed learning (for review, see Smolen et al. 2016; Smith and Scarf 2017). However, the specific intersystem changes with distributed learning are rarely discussed. Here we show that the distributed learning strengthened the memory in several network nodes and reduced vulnerability to brain damage. On our view each episode in distributed learning triggers a new bout of cellular consolidation in regions of the larger network supporting the memory. Each relevant region would come to have a strengthened contribution to the retrieval of the full memory, in the present case to ultimately activate the fear circuitry. A parsimonious account holds that this strengthening occurs without duplication or the transfer of the memory. We propose that distributed learning episodes modify networks involving each of these regions and enhances their modal contribution in such a way that successful recall can occur with activation of a fraction of the initially engaged regions. Damage to any single region would tend to reduce retrieval of fear in the case of memories that are established with relatively weak learning parameters, such as those involved with massed learning, and thus would result in retrograde amnesia. This is congruent with the retrograde amnesia effects observed following damage or inactivation to either the HPC, PRH, and ACC after a single context fear conditioning session (Bucci et al. 2000; Burwell et al. 2004; Frankland et al. 2004; Wiltgen et al. 2006; Sutherland et al. 2008; Broadbent and Clark 2013; Lehmann et al. 2013; Sparks et al. 2013). In contrast, after modifications to the networks during distributed learning of context fear, damage to no single region involved can prevent retrieval of the fear memory. Damage to at least two regions (e.g., HPC and PRH) supporting the fear memory would now be required to induce retrograde amnesia. Thus, with distributed learning episodes, the context fear memory went from requiring all structural network nodes to at least $N-1$ nodes.

Cortical tissue damage has previously been shown to cause impairments in tasks of learning and memory, regardless of where the damage was sustained (Lashley 1931). This has been termed *mass action* and taken as evidence against functional localization (Lashley 1931). It is unlikely, however, that our combined lesions, HPC + PRH or HPC + ACC, caused retrograde amnesia because of the extent of overall tissue damage, rather than because of specific memory system disruption. A mass action account in this study can be reduced by contrasting the lesions sizes and amnesic findings between the two combined lesion experiments (experiments 2 and 3). The PRH is a smaller structure than the ACC (~4.8 $mm^3$ and ~8.2 $mm^3$ posthistology, respectively) and many of our HPC + PRH lesions were smaller in extent than our HPC + ACC lesions (see scatter plots). Given that smaller HPC + PRH damage caused more severe deficits than same size HPC + ACC lesions, a disruption in functional localized contributes is favored over a simple mass action effect.

In conclusion, a single and massed contextual fear conditioning session establishes a long-term memory trace that requires the HPC for retention and/or retrieval of the memory. However, distributing the contextual fear conditioning over several sessions, a procedure known to make memories stronger, also makes the context fear memory become resistant to HPC damage. This finding implies that the memory trace must be more strongly represented in nonHPC networks, at least to an extent that enables successful retention and/or retrieval of the memory. The current study further demonstrates that the PRH and ACC are two structures of the nonHPC network supporting this reinstated context memory. Damaging either structure in combination with HPC damage caused retrograde amnesia. Importantly, this finding also suggests that the HPC does not disengage from the context memory over the course of distributed conditioning, but that a smaller portion of the structural network supporting the memory trace is required to enable successful retention and/or retrieval of the memory.

# Materials and Methods

## General methods and procedures

### *Subjects*

All procedures were approved by the Trent University Animal Care Committee, which follows the standards of the Canadian Council of Animal Care. Adult male Long-Evans rats (Charles River) aged between 3 and 7 mo were used across the experiments. The rats were housed in pairs in individually ventilated cages on a 12-h light–dark cycle (lights on a 7:00 a.m.). They were provided with 25–30 g of rat chow daily and water was available ad libitum.

### *Apparatus*

Two identical conditioning chambers (Ugo Basile) were used across all experiments. The chambers measured 25.4 × 25.4 × 36.5 cm and were made of Plexiglas, with a circular front opening door. The grid floor consisted of 21 metal rods (3-mm diameter), spaced 1.2 cm apart center to center. The conditioning chambers were also individually housed in a sound-attenuating chamber (54.3 × 46.4 × 55.1 cm). Shocks were delivered through the grid floor, which was connected to a shock generator and scrambler (Ugo Basile). The chambers were cleaned using Oxivir Five 16 concentrate (1:16 dilution) before and after each rat underwent conditioning or retention testing. All conditioning and retention testing sessions were video recorded using a webcam placed above the conditioning chamber and connected to a laptop computer.

ANY-maze software (Stoelting) was used to conduct the context fear conditioning. ANY-maze was programed to maintain the internal box light level at 100 lux and fan intensity at 50% during conditioning and testing. The software also quantified the amount of time each rat spent freezing (absence of movement except for breathing) and the program parameters were set at a sensitivity of 70 for freezing onset and 80 for freezing offset. In addition, a freezing bout was only initiated after 250 msec of continuous freezing. From the time spent freezing during the test, the percent time freezing score for each rat was computed and used as an index of learning and memory.

### *Procedures*

***Conditioning.*** The rats were individually transported in a plastic bucket to the testing room and placed inside the conditioning chamber. Foot shock parameters were set at 0.7 mA for 2 sec. At the end of the conditioning session, the rats were removed and immediately transported back to their home cage.

***Surgery.*** The rats were anaesthetized with isoflurane (Abbott Laboratories). They were pretreated with an analgesic (0.02 mL of Metacam at 5 mg/mL, s.c.; Boehringer-Ingelheim) as well as an anticonvulsant (0.4 mL of gabapentin at 100 mg/mL, i.p.; Chiron). The rats were then placed in a stereotaxic frame (Stoelting). An incision was made along the midline of the scalp, which was then retracted to expose the skull and bregma.

For the HPC lesion, small burr holes were drilled at six sites bilaterally and a *N*-methyl-D-aspartic acid and tetrodotoxin cocktail (7.5 µg/µL NMDA + 2 ng/µL TTX, in 0.9% saline; Sigma Chemical) was injected across the HPC (Supplemental Table S1) using a microinfusion pump (KD Scientific). We added TTX to the NMDA because the combination substantially reduces seizure activity associated with the neurotoxic lesions (Sparks et al. 2011). This cocktail was injected into the HPC at a rate of 0.4 µL/min and the volume varied between 0.3 and 0.4 µL depending on the injection site (Supplemental Table S1). The injection needle remained in place for 2 min after each injection to allow diffusion of the NMDA–TTX cocktail. After the completion of all injections, the incision was sutured.

For the PRH lesion, small burr holes were drilled at five sites bilaterally at a 10° lateral angle (see Table 1). The NMDA + TTX cocktail was injected into the PRH at a rate of 0.4 µL/min and a volume of 0.2 µL at each injection site (see Supplemental Table S1), again at a 10° angle.

For the ACC lesion, small burr holes were drilled at 11 sites bilaterally. The NMDA + TTX cocktail was injected into the ACC at a rate of 0.4 µL/min and a volume of 0.2 or 0.4 µL at each injection site (see Supplemental Table S1).

For the Sham lesion, the Sham control rats received the same surgical procedures with the exception that no damage was done to the skull or brain.

For 7 d following surgery, all rats were given an oral analgesic (0.1 mL of Metacam oral suspension at 1.5 mg/mL, p.o.; Boehringer-Ingelheim) daily.

### *Histology*

Following the completion of behavioral testing, the rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (0.3 mL; 340 mg/ml) and perfused intracardially with 200 mL of phosphate-buffered saline followed by 200 mL of 4% paraformaldehyde. The brains were removed and stored in 4% paraformaldehyde for 24 h before being transferred to 0.1% sodium azide/30% sucrose solution to cryoprotect the tissue. The brains remained in the latter solution until sectioning and at the minimum for 48 h. The brains were sectioned at a thickness of 40 µm using a cryostat (Slee). Every twelfth section (sectioning sampling fraction of 1/12) extending through the target structure of interest (HPC, PRH, and ACC) was mounted onto Superfrost Plus glass microscope slides (Fisher Scientific), stained with cresyl violet, and coverslipped. Digital images of each section were then taken at a 2× magnification using a light microscope (Nikon H600L), camera (DS-Qi1Mc), and Nikon Element software (Nikon Instruments, Inc.), in order to enable unbiased/assumption-free stereological quantification of the lesions.

The HPC lesion extent in each rat was estimated according to the Cavalieri and point-counting principles (Mouton 2002). Using ImageJ software (http://rsb.info.nih.gov/ij), a sampling grid with an area per point of 0.05 $mm^2$ was randomly superimposed on each digitized section. The HPC was defined as spanning from −1.72 mm to −6.72 mm relative to bregma (Paxinos and Watson 2007). Grid points that intersected the HPC cell fields (CA1-3, hilus, fasciolarum cinereum, and dentate gyrus) were counted for each section (10–12 sections per brain). The total number of points counted in the HPC for each brain was then divided by the average count from four control rats (experiments 1 and 2: Mean = 443.0, SD = 43.4; experiment 3: Mean = 432.0, SD = 2.58) and multiplied by 100 to produce an estimate of the percent of remaining tissue, the complement of which corresponded to the lesion size.

The point-counting approach was also used to quantify the PRH and ACC lesions. The PRH borders were consistent with those of Burwell (2001). If mapped onto Paxinos and Watson's Rat Brain Atlas (Paxinos and Watson 2007), it spanned from −3 mm to −7.8 mm relative to bregma and encompassed both the PRH and ectorhinal cortex. Using a 0.05 $mm^2$ grid, points intersecting intact tissue within the predefined PRH area were counted for each section (8–10 sections per brain). The average point count from four control rats averaged 528.5 (SD = 37.1). The ACC borders were defined according to Vogt and Paxinos (2012). If mapped onto Paxinos and Watson's Rat Brain Atlas (Paxinos and Watson 2007), it spanned from 5 mm to 0 mm relative to bregma and encompassed the Cg1 and Cg2, prelimbic cortex and infralimbic cortex. The ACC damage was quantified using a grid of 0.1 $mm^2$. The average number of points counted in the ACC for Shams averaged 467.0 (SD = 31.4), with approximately eight to 10 sections per brain.

Volumes of the damaged tissue were also calculated for each structure. These estimates were computed for each rat by multiplying the area per point (grid size; 0.05 $mm^2$ or 0.1 $mm^2$), by the inverse of the section sampling fraction (12), by the average posthistology section thickness (15 µm), and by the point-count difference between the lesion rat and the control group average score. These estimates were used to complete correlational analyses between the absolute lesion size and freezing behavior on the retention tests.

### *Statistical analyses*

Parametric statistical tests were used to analyze the data using SPSS 25 software. Specifically, ANOVAs were used to assess differences across groups, *t*-tests to assess differences between two groups or repeated testing of a group, and correlations to examine whether changes in behavioral measures were associated with lesion size. In all instances, an $\alpha$ level corresponded to 0.05.

## Experiment-specific procedures

### *Experiment 1*

Twenty rats received 10 context–shock pairings within a single 30 min fear conditioning session. Initial shock onset was set at 120-sec, with a recurring shock every 3 min thereafter. The rats were then removed and returned to their home cage. The rats were then matched according to their freezing behavior across the conditioning session and assigned to either the Sham ($n = 8$) or HPC ($n = 12$) group. The conditioning-to-surgery interval was 7–9 d, which corresponded to the same interval used in the later experiments of this study. After the surgical recovery period (9–11 d), the rats were returned to the conditioning context and given a 5-min retention test. No shock was delivered during this test.

### *Experiment 2*

Twenty-four rats received 10 conditioning sessions distributed across five consecutive days. On each day, the rats received a 3-min conditioning session in the morning (10:00–12:00 a.m.) and another 3-min session in the afternoon (1:00–3:00 p.m.). Thus, ∼3 h separated the a.m. and p.m. conditioning sessions for each rat on each day. A session consisted of the rats receiving a single 2-sec shock at the 2-min mark. Immediately after a session, the rats were returned to their home cage.

The rats then either received a Sham ($n = 8$), HPC ($n = 7$) or PRH ($n = 9$) lesion 3–5 d following the final conditioning session (Surgery 1). This corresponds to 7–9 d following the onset of conditioning, matching the conditioning-to-surgery interval in experiment 1. Following the surgical recovery period, the rats were returned to the conditioning context for a 3-min retention test (Retention 1). Note that no shock was delivered during this test.

Three days to 5 d following Retention 1, the rats received a second surgery (Surgery 2). The rats that previously received HPC lesions now received PRH lesions and vice versa for the PRH group. The Sham rats received a second sham surgery. This created two groups: Sham ($n = 8$) and HPC + PRH ($n = 16$).

Nine days to 11 d after Surgery 2, the rats were returned to the context for a 7-min test. The first 5 min of the test served as the second retention test, whereas the remaining 2 min served as a reconditioning session involving two context–shock pairings. Specifically, the rats received a shock at the 5- and 6-min mark. This reconditioning session was given to determine, in the event of a retention deficit, whether contextual fear conditioning could be reacquired and address concerns of reduced freezing behavior being attributed to a performance deficit (i.e., the inability to engage in freezing). One day after Retention 2, the rats were returned to the conditioning context for a final 3-min retention test (Retention 3) to assess memory for the reconditioning.

### *Experiment 3*

This experiment involved the same experimental design and procedures as in experiment 2 (see Fig. 4) with the exception that the ACC was damaged rather than the PRH. In addition, duration of the first retention test (Retention 1) was 5 min rather than 3 min. The *n* for the Sham, HPC, and ACC were 4, 6, and 8, respectively, at the time of Retention Test 1, whereas it was 4 for the Sham group and 14 for the HPC + ACC group for Retention 2.

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