Spaced Learning Parameters for HPC-Independent Context Fear Memory

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Abstract

Spaced Learning Parameters for HPC-Independent Context Fear Memory

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Damage to the hippocampus (HPC) causes retrograde amnesia for some memories, but spaced learning mitigates this. Contextual fear conditioning (CFC) studies in rats demonstrate that distributing conditioning over multiple sessions makes a memory less vulnerable to HPC damage, and it has been suggested this occurs through incremental strengthening of the memory outside the HPC via separate bouts of cellular consolidation. To explore this, we examined the number of, and temporal intervals between, spaced CFC sessions required to make a memory less vulnerable to HPC damage. Experiment 1 established six sessions spaced over three days as sufficient to create a memory no longer requiring the HPC. Experiments 2 and 3 found that spacing those six sessions in a single day also created a memory no longer requiring the HPC, but only when the sessions were separated by an interval believed to be sufficient for separate bouts of cellular consolidation to occur.

Keywords: hippocampus, spaced learning, memory, retrograde amnesia, context fear, consolidation

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1. General Introduction

Distributing learning over multiple spaced sessions produces stronger long-term memory than a single massed learning session, a phenomenon known as the *spacing effect* or *spaced learning*. This was first demonstrated in 1885 by Hermann Ebbinghaus, who found that the number of study sessions required to retain a 12-nonsense syllable series was reduced by nearly half when the learning was distributed over three days rather than in a single day (Ebbinghaus 1913). Since then, the spacing effect has become a widely replicated phenomenon in humans and non-human animals as well as across a variety of memory tasks (Kornmeier and Sosic-Vasic 2012).

When we compare spaced learning to massed learning and observe that spaced learning results in stronger memories, it implies that the memory trace created by spaced learning must be different, in some way, than the one created by massed learning. Support for this view comes, in part, from studies examining the retrograde amnesic effects of hippocampal lesions (Lehmann et al. 2009; Lehmann and McNamara 2011; Lehmann et al. 2021; Shepherd et al. 2021). For instance, Lehmann et al. (2009) demonstrated that lesions of the hippocampus (HPC) that typically cause retrograde amnesia for contextual fear conditioning (CFC) do not cause amnesia if the conditioning was spaced across several days

rather than in a single massed learning session. Hence, the contextual fear memory trace was resistant to HPC damage in the spaced condition, and therefore different than the memory trace acquired in a single session. This change is argued to be caused by incremental cellular consolidation bouts (Sutherland et al. 2010; Shepherd et al. 2021), processes during which memories are stabilized within memory systems through protein synthesis and the updating of synaptic connections (Dudai, 1996; Mednick et al., 2011). Ultimately, the spaced bouts would strengthen and/or expand the initial trace in nonHPC memory systems, such as the perirhinal and anterior cingulate cortices (Shepherd et al. 2021), and make it resistant to HPC damage (Sutherland et al. 2010). Specifically, Sutherland et al.'s (2010) Distributed Reinstatement Theory proposes that each repetition of the learning episode or reinstatement of the memory induces a new cellular consolidation bout and incrementally strengthens the memory trace in the nonHPC system.

Landauer (1969) was among the first to suggest that cellular consolidation processes could account for the memory benefits conferred by spaced learning. More recent accounts suggest that consolidation and reconsolidation processes enhance the trace (Smith & Scarf, 2017) and that the cellular mechanisms during the bouts can differ depending on whether they pertain to the first learning

episode (initial consolidation) or the following ones in the spaced sequence (reconsolidation) (Lee, 2008; Lee and Hynds, 2013). For instance, Lee (2008), by pharmacologically inhibiting protein synthesis, found that, in the HPC, brain derived neurotrophic factor (BDNF) was essential for cellular consolidation associated with initial contextual fear conditioning, but not cellular reconsolidation associated with a second conditioning session given a day later. Lee (2008) further demonstrated that the reconsolidation, but not the consolidation, is dependent on the immediate early gene Zif268 (also known as EGR-1) in the HPC. This double dissociation convincingly suggests that different cellular mechanisms contribute to the development of a memory trace over spaced learning. It is important to note, however, that there is also overlap between consolidation and reconsolidation processes (Bozon et al. 2003; Lee and Hynds 2013). Regardless, the point remains that spaced learning provides opportunity for increased cellular consolidation/reconsolidation bouts, resulting in a stronger memory trace. For this study, however, we will not distinguish between consolidation and reconsolidation.

The interval between learning events is of importance in spaced learning (Parsons and Davis 2012; Kelley and Whatson 2013) with very short intervals (seconds to a few minutes) not benefiting long-term memory as much as intervals

of a few hours (Aziz et al., 2014; Tintorelli et al., 2020). At a neural level, this can be explained by considering whether the intervals between learning events are sufficient to enable new protein synthesis. Cellular processes associated with learning are viewed to have refractory periods and two learning episodes too closely paired in time would not enable consolidation and plasticity summation to increase the memory trace (Smolen et al. 2016). Cellular consolidation can last up to 100 hours (Sutherland et al., 2010), but in the rat a new learning event as soon as 60 minutes after the first can lead to additional processes of cellular consolidation and benefit long-term memory (Tintorelli et al., 2020). Intervals shorter than 15 minutes between learning events, however, are less likely to result in the same benefit (Tintorelli et al., 2020). Additionally, examination of long-term potentiation (LTP), a form of synaptic plasticity, in rat HPC slices suggests that spaced theta stimulation bouts are more likely to enhance LTP and plasticity at intervals of an hour or more, but fail with shorter intervals (Kramar et al., 2012). Hence, spaced learning, with intervals of an hour or more, can induce incremental cellular consolidation and plastic changes in the rat. These changes could support the development, over spaced learning, of a sufficiently strong context fear memory trace to no longer depend on the HPC. In contrast, massed contextual fear conditioning or conditioning with short spacing intervals (<15 min)

would result in overlapping cellular consolidation bouts and fail to sufficiently strengthen the memory trace in nonHPC systems to make it HPC independent.

The goal of this study was to examine whether incremental consolidation bouts, resulting from spaced learning, indeed make a context fear memory become resistant to HPC damage. In this study, we did not manipulate cellular consolidation processes using protein synthesis inhibitors or other pharmacological compounds. Rather we manipulated the interval between conditioning sessions to reduce the opportunity for separate and incremental consolidation bouts. In the first experiment, we established the minimum number of spaced context fear conditioning sessions required to make a memory independent on the HPC following the paradigm described by Lehmann et al. (2009). We found that as few as six single shock sessions distributed across three days was sufficient to make a context fear resistant to complete HPC lesions. In the second experiment, we examined whether spacing the six conditioning sessions by an interval of 1-hr, one that should still provide opportunity for incremental bouts of cellular consolidation, would result in the same spared memory following complete HPC lesions. We found that, indeed, the memory trace became sufficiently strong in this instance to withstand the lesions. In the third experiment, the interval between the six conditioning sessions was reduced

to five minutes, greatly reducing the opportunity for incremental consolidation bouts, and found that the memory remained dependent on the HPC.

2. Experiment 1

Spaced learning can make a memory more resistant to HPC damage in a variety of tasks (Lehmann et al. 2009; Sutherland et al. 2010; Lehmann et al. 2021). This suggests that spaced learning alters the memory trace and creates one that no longer requires the HPC (the memory becomes *HPC-independent*). This has been repeatedly demonstrated in contextual fear conditioning with 6-11 conditioning sessions spaced over 3-6 days (Lehmann et al. 2009; Gulbrandsen et al. 2013; Shepherd et al. 2021). In contrast, massed conditioning or spaced learning involving two or three spaced sessions insufficiently strengthens the trace in the nonHPC and do not prevent the retrograde amnesic effects of HPC lesions or inactivations in this task (Broadbent and Clark 2013; Gulbrandsen et al. 2013; Lehmann et al. 2013; Sparks et al. 2013). The transition from HPC dependent to independent is argued to be an incremental effect occurring over the course of the spaced learning sessions, and six sessions over three days seems to be key in establishing a strong enough trace to no longer require the HPC. The current experiment aimed to determine, in our laboratory, the number of spaced contextual fear conditioning sessions required to make the memory HPC-

independent, and whether the retention pattern across the spacing condition supports an incremental transition. The design of the experiment is illustrated in Figure 1 in which rats were given between 2 and 10 spaced contextual fear conditioning sessions with a set interval prior to receiving complete HPC lesions and being tested for retention.

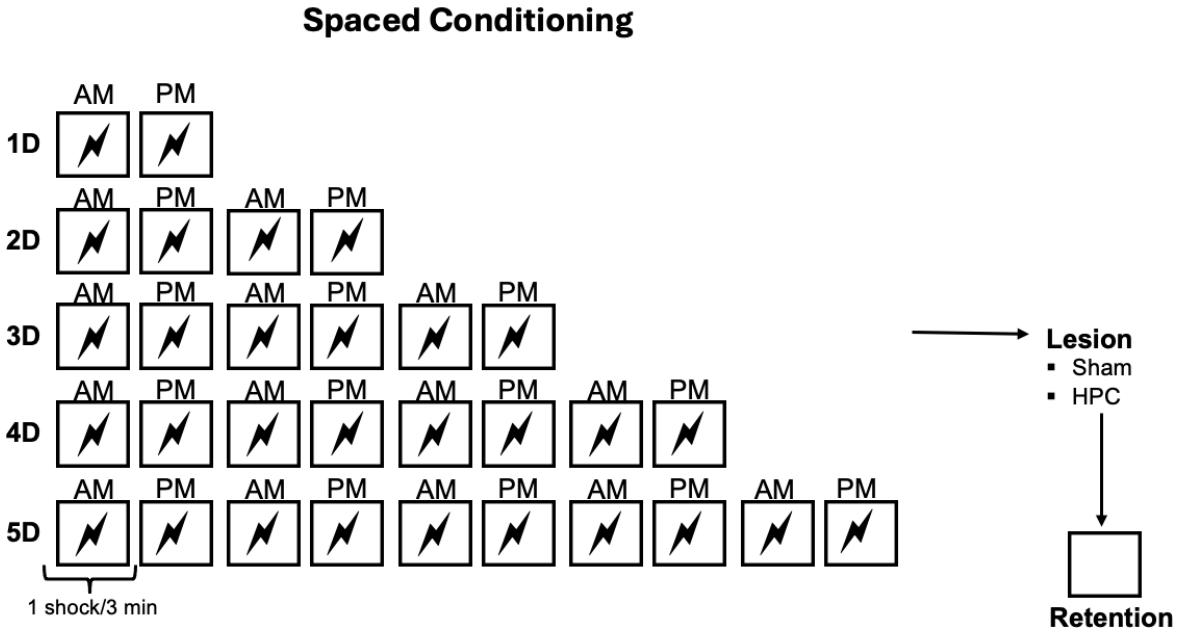

Figure 1. Rats received two contextual fear conditioning sessions per day for 1 to 5 days. The rats then received Sham or HPC lesions and were tested for retention 11-14 days later.

2.1 Methods

2.1.1 Subjects

All procedures were approved by the Trent University Animal Care Committee, under the guidelines of the Canadian Council on Animal Care. The

subjects were 102 Male Long Evans rats (Charles River, Quebec, Canada), weighing 300g-350g and aged 3-4 months at the beginning of their behavioural training. Rats were housed in pairs in standard laboratory cages and maintained on a 12-hour light-dark cycle (lights on 7:00am, off 7:00pm). Each rat received 25–30g of rat chow daily and had access to water *ad libitum*.

2.1.2 Apparatus

Two identical Ugo Basile (Varese, Italy) conditioning chambers made of Plexiglass and measuring 25.4cm x 25.4cm x 36.5cm were used for all behavioural training and testing. The grid floor of the chambers was included 21 (3mm in diameter) metal rods spaced 1.2cm apart and connected to a shock generator and scrambler (Ugo Basile, Varese, Italy). The conditioning chambers were each housed in two separate, identical sound-attenuating chambers measuring 54.3cm x 46.4cm x 55.1cm. Prior to conditioning and testing of each rat, the chambers were cleaned with an Oxivir Five 16 Concentrate solution at 1:16 dilution. The ANY-maze software was programmed to maintain an internal light intensity of 100 lux inside the boxes during conditioning and retention testing, as well as a constant fan intensity of 50%.

The ANY-maze software (Stoelting, Wood Dale, IL) quantified the amount of time each rat spent freezing during conditioning and retention testing via

webcams mounted on top of each of the plexiglass conditioning chambers and connected to a laptop computer. To measure freezing, the ANY-maze program parameters were set to a sensitivity of 70 freezing onset and freezing 80 for offset. The program considered a rat to be freezing only after they exhibited 250ms of continuous freezing behaviour. The percentage of time spent freezing for each rat was calculated and used as a measure of memory.

2.1.3 Procedure

2.1.3.1 Conditioning

The rats were assigned to receive fear conditioning over one to five days, referred to as the 1D, 2D, 3D, 4D, and 5D groups. The rats in the 1D group received two spaced CFC sessions, one in the morning (beginning at 10:00AM) and another in the afternoon (beginning at 1:00PM). For the rats in the 2D group, the procedure was repeated on the following day, and so forth for the 3D, 4D, and 5D groups. Hence, the rats received two conditioning sessions per day, resulting in a total of 2 sessions for the 1D group, 4 for 2D, 6 for 3D, 8 for 4D, and 10 for 5D, distributed evenly across the respective number of consecutive days.

For each conditioning session, the rats were individually transported in identical 24.0cm x 24.3cm x 33.6cm plastic carriers from their home cage directly to the fear conditioning room, where they were immediately placed inside their

individual conditioning chamber for 3 minutes. At the 2-minute mark, the rats received a 2-second 1.0 mA (1.4 mA peak, 1.0 mA avg) foot shock. At the end of each conditioning session the rats were immediately returned to their home cage.

2.1.4 Surgery

Four to 10 days following their first conditioning session the rats received either Sham or HPC lesion surgery. The rats were anaesthetized with isoflurane (Abbott Laboratories, Chicago, IL) in 0.8 L/min oxygen (Benson Medical Industries, Markham, Ontario) at 14.7 PSIA at 21˚C. They additionally received an analgesic (Metacam, 0.02 ml; 5 mg/ml, s.c.; Boehringer-Ingelheim, Rhineland-Palatinate, Germany). The rats were then placed in a stereotaxic frame (Stoelting, Wood Dale, IL) and an incision was made along the midline of the scalp, which was retracted to expose the skull and bregma. For the HPC lesions, six small burr holes were drilled over each hemisphere and a neurotoxic cocktail solution of N-methyl-D-aspartic acid (NMDA; 7.5 µg/µl, in 0.9% physiological saline, Sigma Chemical, St. Louis, MO) and tetrodotoxin (TTX: 1ng/ul) was injected at seven sites in the HPC of each hemisphere using a 30 Ga needle attached to a 10 µl Hamilton syringe via polyethene tubing (PE-50) (see Table 1 for coordinates). The NMDA+TTX cocktail produces extensive neuronal death and minimize the brief seizure activity period that typically follows neurotoxic lesions (Sparks et al., 2011). The injection rate at

each site was 0.4µl/min and controlled by a KD Scientific microinfusion pump (Holliston, MA). The volume varied between 0.3 ul and 0.4 ul per site and is specified in Table 1. The injection needle remained in place for 2-minutes after each injection to allow diffusion of the NMDA-TTX cocktail. After the completion of all injections, the incision was stitched with suture. The Sham rats received the same surgical procedures with the exception that no damage was made to the skull or brain. For 5 days following surgery, all rats were given an oral analgesic (Metacam, Oral Suspension 0.1 ml; 1.5 mg/ml, p.o.; Boehringer-Ingelheim).

Table 1. Neurotoxic Lesion Injection Coordinates.

Anteroposterior (AP)	Mediolateral (ML)	Dorsoventral (DV)	Infusion volume (µL)
-3	±1.5	-3.6	0.3
-4	±3.0	-4	0.3
-4.9	±3.0	-4	0.3
-4.9	±5.2	-7.2	0.3
-5.7	±4.4	-4.4	0.3
-5.7	±5.4	-7.3	0.4
-5.7	±5.4	-6	0.4

2.1.5 Retention

Eleven to 14 days following surgery the rats were returned to their contextual conditioning chamber for 5-minute retention test. No shock was

administered during this test and freezing behaviour was used as an index of memory.

2.1.6 Histology

Following the completion of behavioural testing, the rats were euthanized with an intraperitoneal injection of sodium pentobarbital (0.5 mL; 340 mg/ml) and perfused intracardially with 300-500mL of 0.9% phosphate-buffered saline followed by 300-500mL of 4% paraformaldehyde. The brains were removed and stored in 4% paraformaldehyde for 24-hours before being transferred to 0.1% sodium azide/30% sucrose solution to cryoprotect the tissue. The brains remained in the 0.1% sodium azide/30% sucrose solution for a minimum of 48-hours until sectioning. The brains were then sectioned at a thickness of 40µm using a cryostat (Slee, Mainz, Germany). Every twelfth section (sectioning sampling fraction of 1/12th) extending through the HPC was mounted onto Superfrost Plus glass microscope slides (Fisher Scientific, Hampton, NH), stained with cresyl violet, and cover slipped. The HPC was operationally defined as spanning from -1.72 mm to -6.72 mm relative to Bregma (Paxinos & Watson, 2006). Digitized images of theses sections were taken at a 2X magnification using a light microscope (Nikon H600L), camera (DS-Qi1Mc), and Nikon Element software (Nikon Instruments Inc., Melville, NY).

The HPC lesion extent in each rat was then estimated according to the Cavalieri and point-counting principles (Schmitz & Hof, 2005). Specifically, 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. Each point that intersected with HPC cell fields (CA1-3, hilus, faciolarumcinereum, and dentate gyrus) was then counted. The total number of points counted for each brain was then divided by the average count from 3 control rats (M=375.33) and multiplied by 100 to produce an estimate of the percent of remaining tissue, the complement of which corresponds to the lesion size.

2.1.7 Statistical Analyses

JASP version 18.3 was used for all analyses and an alpha level of 0.05 was used for significance.

2.2 Results

2.2.1 Histology Results

The behavioural data of HPC rats with a lesion encompassing less than 60% of the HPC in either hemisphere were excluded from the data analyses because lesions smaller than this criteria may fail to cause retrograde amnesia in this task (Scott et al., 2016). In total, the data from 13 HPC rats were excluded from the

experiment (2, 7, 2, and 2 from the 1D, 2D, 3D, and 4D respectively), resulting with 8-10 rats per group.

Briefly, the NMDA-TTX injections produced extensive cell death in both the dorsal and ventral regions of the HPC. The lesions were bilateral and encompassed the CA fields, the dentate gyrus, and extended into the anterior part of the subiculum. Damage outside the HPC was minimal and localized to the injector tracks above the HPC injection sites. The average HPC lesion size for each group is displayed in Figure 2 and a one-way ANOVA revealed a significant difference amongst these groups, $F(4,46) = 3.56$, $p = .0136$. A post-hoc LSD found that the mean lesion size for the 2D HPC group (81.14%) was significantly smaller than the 1D (87.39%) and 5D (91.61%) groups (*ps* < .05), but there were no other significant differences in lesion size between the groups (*ps* > .05).

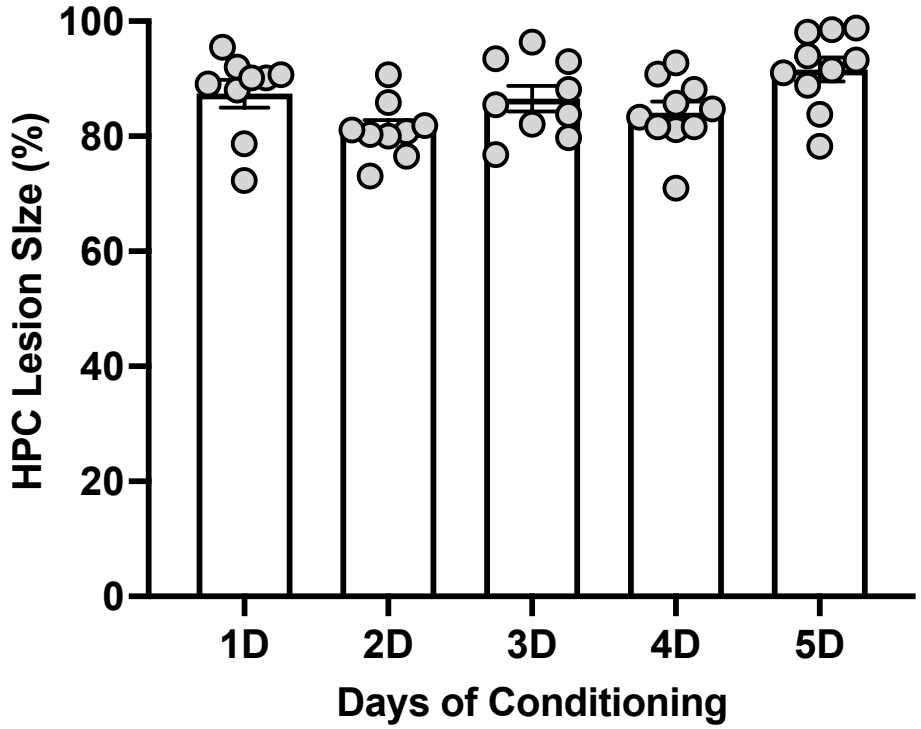

Figure 2. Mean (+/- SEM) HPC lesion size for each of the lesion groups. The 2D group had smaller lesions than the 1D group ($p < .05$), but no other statistically significant differences were found.

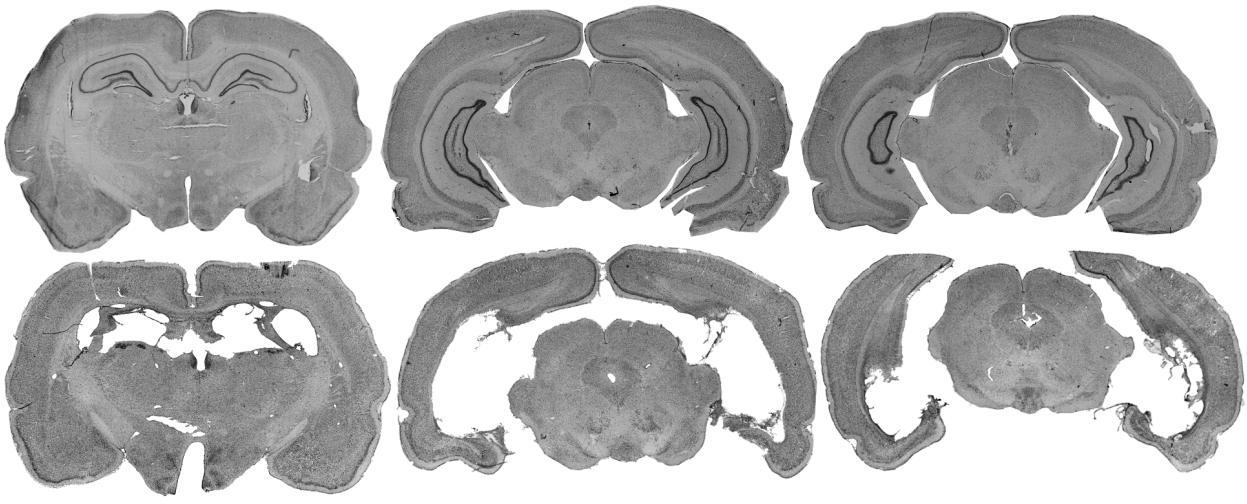

Figure 3. (Top) Photomicrographs of coronal sections throughout the HPC of a control rat. (Bottom) Corresponding photomicrographs from a rat with 84.9% damage to the HPC cell fields.

2.2.2 Behavioural Results

2.2.2.1 Conditioning

Following conditioning, the rats from each spaced learning condition were matched for their freezing behaviour and assigned to either the Sham or HPC surgery condition. The matching aimed to ensure that any potential post-operative retention differences were not due to differences in pre-operative conditioning freezing levels. Specifically, the 1D rats were matched on their pre-shock freezing from their two conditioning sessions, whereas the others were matched on their pre-shock freezing from the four sessions in their final 2 days of conditioning. An ANOVA with Surgery (Sham, HPC) and Acquisitions Days (1D, 2D, 3D, 4D, 5D) revealed no main effect of assigned surgery condition, $F(1,79) = 0.0011$, $p = .974$, suggesting the Sham and HPC groups were matched properly. There was a main effect of Acquisition Days, $F(4,79) = 47.13$, $p < .001$, with post hoc LSD indicating that the 1D rats showed significantly less pre-shock freezing (16.97%) than all other groups, and that the 2D rats showed significantly less pre-shock freezing during acquisition (43.83%) than the 3D, 4D, and 5D rats (*ps* < .05). These differences are consistent with findings that increased conditioning results

in more freezing (Lehmann et al., 2013, Gulbrandsen et al., 2013), and therefore do not undermine the successful matching of the learning of the HPC and Sham groups in each spaced learning condition.

2.2.2.2 Retention

The percentage of time spent freezing during the retention test is displayed in Figure 4. A two-way ANOVA with Surgery (Sham, HPC) and Acquisition Days (1D, 2D, 3D, 4D, 5D) as between-group factors revealed a significant main effect of Surgery, $F(1, 79) = 39.80$, $p < .001$, indicating that HPC rats froze significantly less than the Sham control rats. There was also a significant main effect of Acquisition Days, $F(4, 79) = 35.36$, $p < .001$, indicating that rats receiving a fewer number of spaced conditioning sessions (1D, 2D) froze less on the retention test. These main effects were not further analyzed because the Surgery x Acquisition Days interaction was significant, $F(4, 79) = 21.08$, $p < .001$, indicating that the effect of Surgery on retention varied with the spaced conditioning.

Analyses of the interaction with Least Significant Difference pairwise comparisons indicated that the HPC rats from the 1D and 2D groups froze significantly less than their respective Sham control group ($ps < .05$). In contrast, the HPC rats from the 3D, 4D, 5D groups did not freeze significantly less than their respective Sham control groups ($ps > .05$). These comparisons suggest that a

context fear memory can become independent of the HPC with as few as six conditioning sessions spaced over three days.

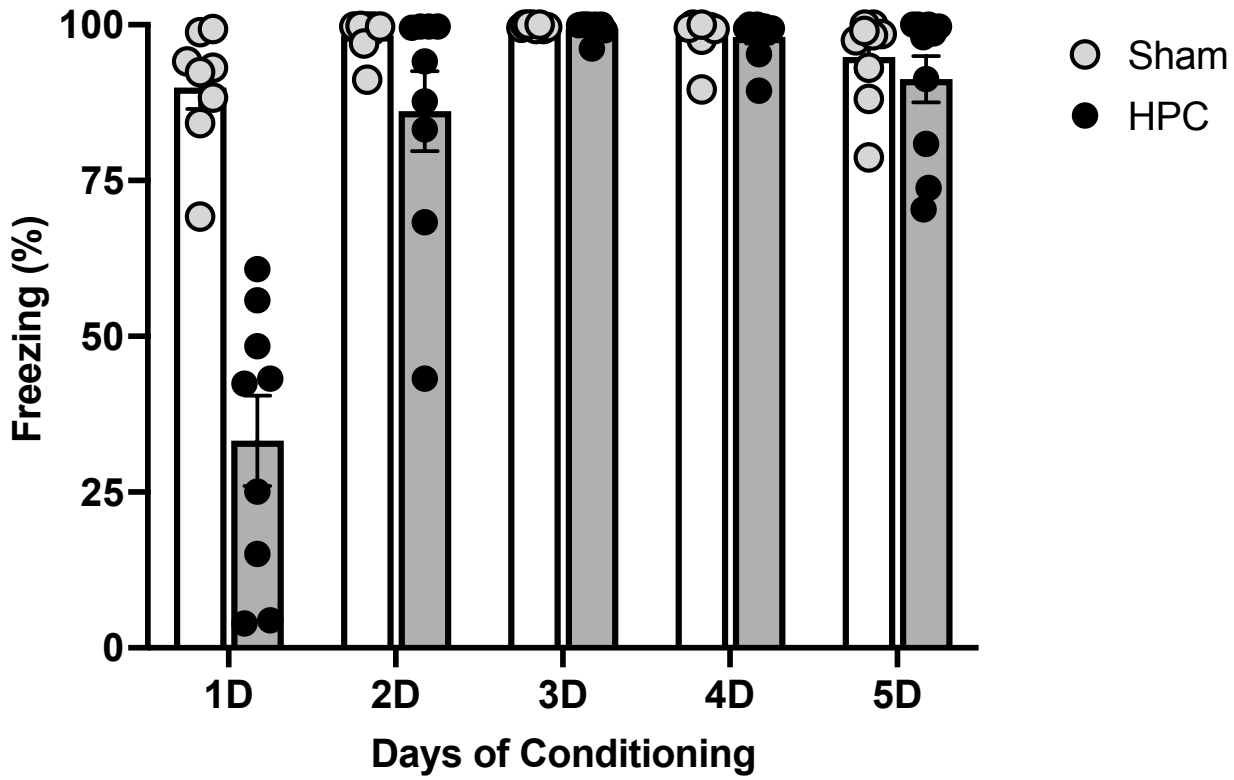

Figure 4. Mean (+/-SEM) percent time freezing by Sham and HPC rats during the retention test in Experiment 1. The HPC rats in the 1D and 2D groups froze significantly less than their respective Sham control groups (*ps* < .05), suggesting that the HPC damage caused retrograde amnesia in these rats and that four spaced conditioning sessions is insufficient to create an HPC-independent context fear memory. In contrast, the HPC rats in the 3D, 4D and 5D groups did not freeze significantly less than the rats in their respective control group (*ps* > .05), suggesting that these HPC rats did not suffer from retrograde amnesia and that as few as six spaced conditioning sessions results in a HPC-independent context fear memory.

2.3 Discussion

We found that as few as six contextual fear conditioning sessions spaced over three days are sufficient to make a context fear memory independent of the HPC. Specifically, the HPC rats that received two or four conditioning sessions froze significantly less than their respective control groups during the retention test, which indicates retrograde amnesia and that the HPC still supports the context fear memory. In contrast, those that received six or more sessions froze as much as their respective control groups, suggesting that as few as six spaced conditioning sessions can make a strong context fear memory trace in the nonHPC system to support retention and expression of the memory. The findings of the current experiment align with findings from previous studies that have reported the effectiveness of spaced learning in forming a context fear memory resistant to complete HPC damage (Lehmann et al. 2009; Lehmann and McNamara 2011; Lehmann et al. 2021; Shepherd et al. 2021)

The transition of the context fear memory from HPC-dependent to HPC-independent through spaced learning could take several forms. One possibility is that it occurs incrementally, and another is that there is a learning threshold that, when met, causes the memory to suddenly 'switch' to a non-HPC memory trace. The data across the lesion groups that received two to six spaced conditioning

sessions, however, do not support this threshold possibility. Rather, the distribution of means supports an incremental relationship with a plateau reached after six conditioning sessions. Hence, spaced learning appears to progressively strengthen the non-HPC memory trace until the HPC is no longer required to support the memory.

3. Experiment 2

The previous experiment demonstrated that a context fear memory could become progressively resistant to HPC damage in as few as six spaced conditioning sessions distributed across three days. Sutherland et al. (2010) argues that each of these sessions induces a new cellular consolidation bout and incrementally strengthens the memory trace in the nonHPC system. If this is the case, then providing the six conditioning sessions with a spacing interval between conditioning sessions to allow new and separate consolidation bouts should result in an equally HPC-independent context fear memory. Tintorelli et al. (2020) demonstrated that one hour between learning events should be sufficient for separate cellular consolidation bouts that can increase a memory trace. Accordingly, we examined whether six spaced conditioning sessions, each spaced by an hour, would make an HPC-independent memory. Importantly, this would be the first demonstration of a context fear memory becoming HPC independent

after learning in a single day and, to our knowledge, only the second instance of a memory transitioning from HPC dependent to independent in such a short period (Tse et al., 2007). The experimental design also included a condition in which the rats received the six spaced conditioning sessions over three days to replicate the observed findings of the 3D condition from Experiment 1. In addition, a third group of rats receive massed conditioning, meaning all six context-shock pairings in a single session, was included in the experiment to confirm that spacing of the conditioning and associated consolidation bouts result in the HPC-independent memory and not solely the number of context-shock pairings. The experimental design is illustrated in Figure 5.

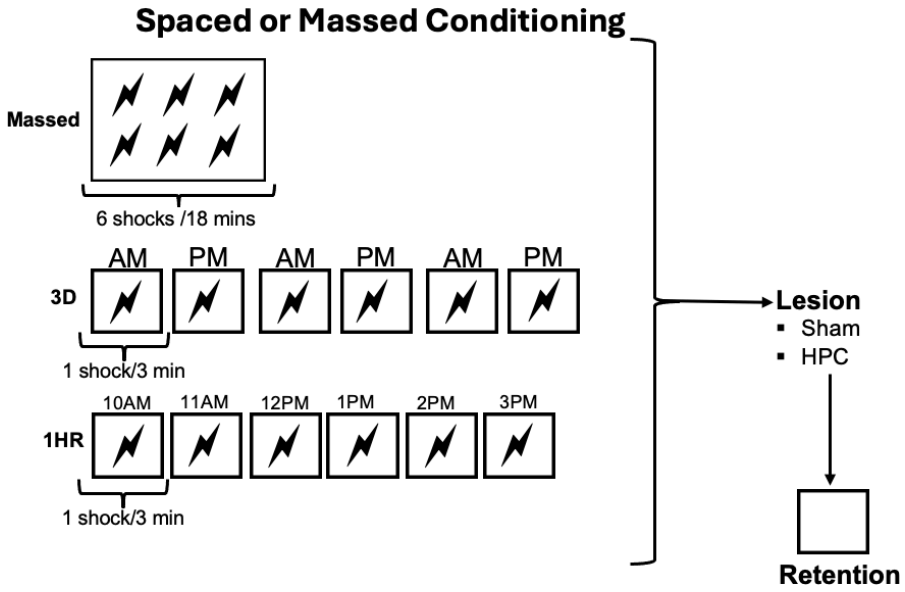

Figure 5. Rats received six context-shock pairings in total, either massed within one session or spaced over three days or one day. Rats were returned to their home cages between spaced sessions. The rats then received Sham or HPC lesions and were tested for retention 11-14 days later.

3.1 Methods

3.1.1 Subjects

The subjects were 52 male Long Evans rats, housed under the same conditions as those in Experiment 1.

3.1.2 Procedures

Following the conditioning parameters from Experiment 1, all rats received six context-shock pairings, but spaced differently. Specifically, the rats either received their conditioning in a single massed session of 6 shocks administered over 18 minutes separated by 3 minutes each (*Massed* condition), a spaced learning condition of 2 single-shock sessions per day for 3 days (*3D* condition, as in Experiment 1), or a spaced learning condition of 6 single-shock sessions in one day, separated by an hour between sessions (1HR condition). Note that all rats from the spaced conditions were returned to their home cage between sessions. Four to seven days following completion of the conditioning, the rats receive sham or HPC lesions and were tested for retention as described in the previous experiment. Histological analyses of the lesions also followed the procedures described in Experiment 1.

3.2 Results

3.2.1 Histology

The data from 12 HPC rats were excluded from the experiment for not meeting the minimum HPC lesion size criteria (8, 1, and 3 from the Massed, 3D, and 1HR respectively), resulting in 5-9 HPC rats per group. The average HPC lesion size for each group is displayed in Figure 6, and a one-way ANOVA found no significant difference in HPC lesion size between, $F(2,18) = 0.02$, $p = 0.9812$.

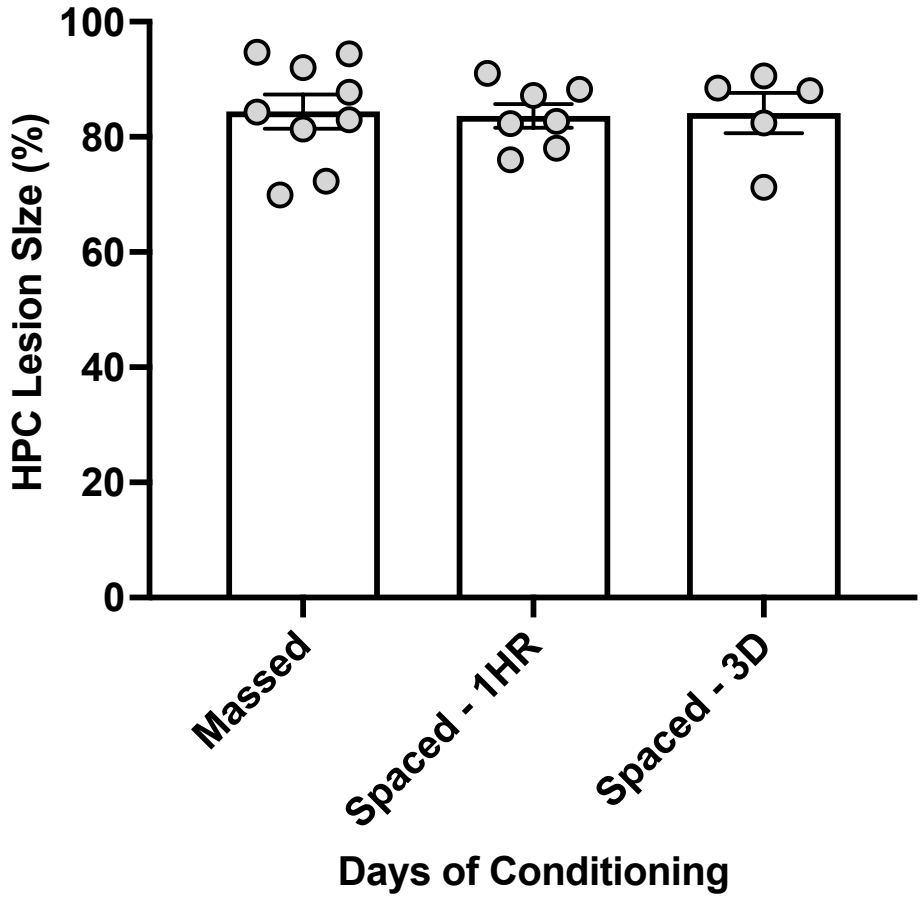

Figure 6. Mean (+/-SEM) lesion % of the HPC lesion rats in all groups, no significant difference was found in lesion size between the groups (*ps* > .05).

3.2.2 Behavioural Results

3.2.2.1 Conditioning

Following conditioning, the rats from each learning condition were matched for their freezing behaviour and assigned to either the Sham or HPC surgery condition. As in the previous experiment, rats in the spaced learning conditions (1HR, 3D) were matched on the pre-shock freezing from their final 4 conditioning sessions. An ANOVA with Surgery (Sham, HPC) and Spaced Learning Interval (1HR, 3D) on pre-shock freezing during acquisition revealed no main effect of assigned surgery condition, $F(1,18) = 0.023$, $p = .882$, indicating that there was no significant difference in pre-shock freezing levels between rats assigned to the Sham or HPC lesion group in the spaced learning condition. Because the rats in the Massed condition received just the one long conditioning session and could therefore not be matched the same way as the rats that received spaced learning, they were matched based on their total freezing during conditioning and assigned to either the Sham or HPC lesion condition, and these conditions did not significantly differ from one another $t(16) = 0.335$, $p = .742$, in their freezing during acquisition.

3.2.2.2 Retention

An ANOVA with Surgery (Sham, HPC) and Learning Condition (Massed, 3D, 1HR) as between-group factors revealed significant main effects of Surgery, $F(1, 34) = 6.13$, $p = .018$, and Learning Condition, $F(2, 34) = 11.83$, $p < .001$. These main effects were not further analyzed because a significant Surgery x Learning Condition interaction was found, $F(2, 34) = 7.18$, $p = .003$, indicating that the effect of Surgery on retention varied with the way learning occurred. Specifically, pairwise comparisons using Least Significant Difference indicated that the freezing of the Massed HPC rats was significantly lower than that of the Massed Sham rats ($p < .001$), suggesting that that the HPC damage caused retrograde amnesia for massed learning. However, 3D HPC did not significantly differ from 3D Sham ($p = .886$), and 1HR HPC was also not significantly different from 1HR Sham ($p = .866$), suggesting that HPC lesions did not cause retrograde amnesia for the memories acquired via spaced learning. Moreover, Massed HPC rats froze significantly less than both 3D HPC and 1HR HPC rats ($ps < .001$), whereas 1HR HPC was not significantly different from 3D HPC ($p = .320$), suggesting that an hour between spaced learning trials is sufficient to create an HPC-independent memory.

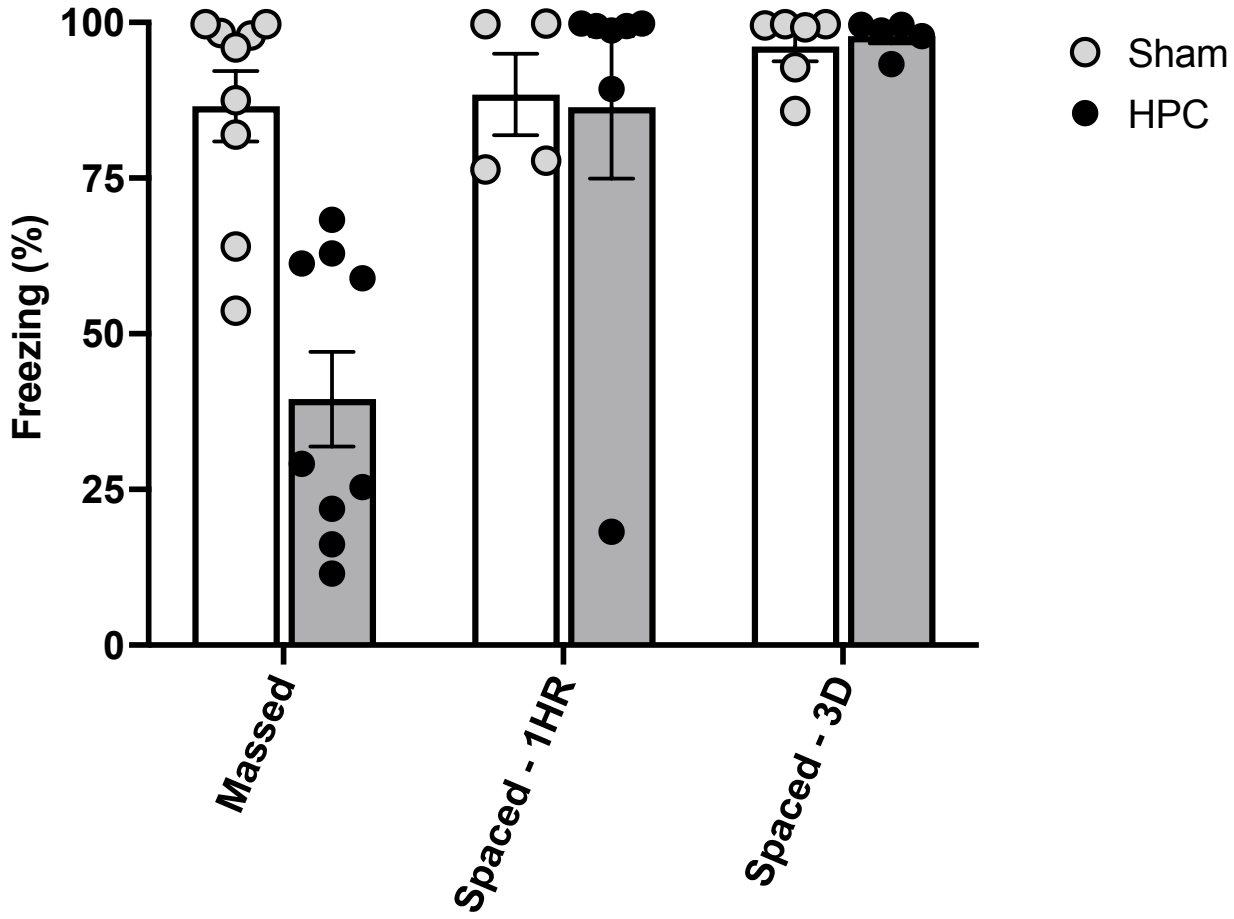

Figure 7. Mean (+SEM) percent time freezing by Sham and HPC rats during the Experiment 2 retention test. The HPC rats in the Massed group froze significantly less than their respective Sham control rats ($p < .001$) suggesting that the HPC damage caused retrograde amnesia in these rats. The HPC rats in the 1HR and 3D groups did not freeze significantly less than the rats in their respective Sham control groups (*ps* > .05), suggesting that these HPC rats did not suffer from retrograde amnesia and that 6 spaced conditioning sessions results in an HPC-independent context fear memory whether distributed across 3 days or within a single day and separated by 1 hour.

3.3 Discussion

The aim of the present experiment was to explore cellular consolidation theories of spaced learning by manipulating the inter-sessions interval within a

spaced learning condition to all fall within one day, with sufficient time for cellular consolidation to occur between learning events. Whereas rats that received all 6 learning events in one massed session suffered retrograde amnesia following HPC lesion surgery, rats that received their 6 learning sessions spaced over 3 days or spaced across 1 day with an hour interval between did not show evidence of retrograde amnesia. The results of the present experiment suggest that creating a long-term memory trace for a contextual fear memory outside the HPC, a process postulated to necessitate days (Kim and Fanselow, 1992) can be accomplished in a single day if learning is separated by an hour. As one hour is a sufficient temporal window for incremental bouts of cellular consolidation to occur, these findings therefore lend support to cellular consolidation theories of spaced learning.

4. Experiment 3

The previous experiments demonstrated that spacing the six contextual fear conditioning sessions in a single day with a sufficiently long interval for new and separate consolidation bouts to occur creates a memory independent of the HPC. If we take this as suggestion that spaced learning is supported by separate bouts of cellular consolidation, then providing the six conditioning sessions with a spacing interval insufficient for, or at least limiting, new separate bouts of cellular

consolidation to occur should result in retrograde amnesia following HPC-lesion. Intervals shorter than 15 minutes between learning events would result in overlapping cellular consolidation bouts (Kramar et al., 2012; Smolen et al. 2016; Tintorelli et al., 2020), so, in the present experiment, we spaced the six sessions with 5-minute intervals between sessions. The experimental design, illustrated in Figure 8, also included a condition in which the sessions were separated by one-hour intervals to replicate the observed findings of the 1HR condition from Experiment 2.

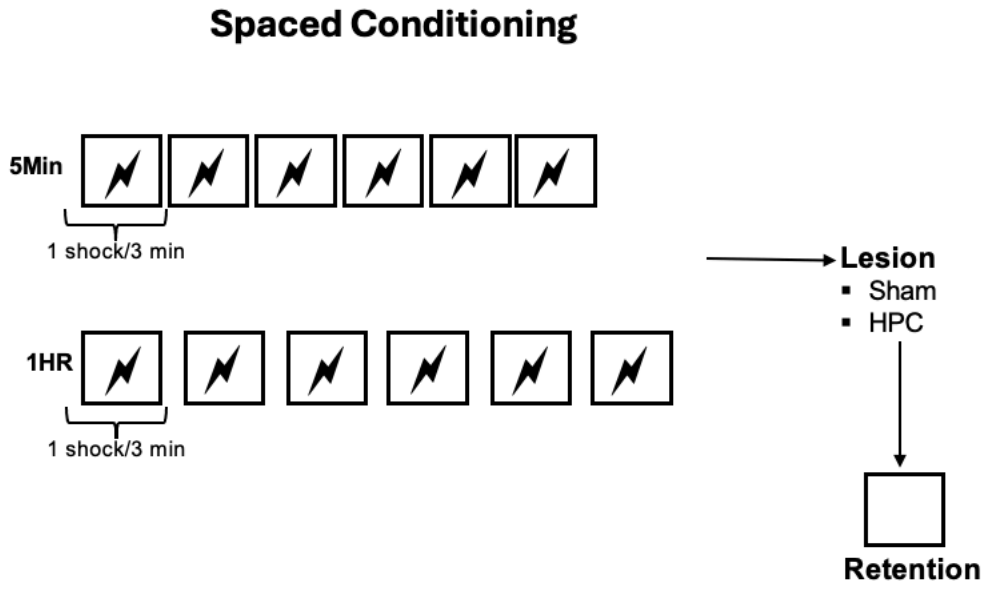

Figure 8. Rats received six context-shock pairings spaced over one day. In once condition the interval between sessions was 5 minutes (5Min) whereas it was 1 hour for the other (1HR). The rats then received Sham or HPC lesions and were tested for retention 11-14 days later.

4.1 Methods

4.1.1 Subjects

The subjects were 33 male Long Evans rats, housed under the same conditions as those in Experiments 1 and 2.

4.1.2 Procedures

Following the conditioning parameters from the previous two experiments, all rats received six context-shock pairings, but spaced differently. Specifically, the rats received spaced conditioning of 6 single-shock sessions in one day, separated by either an hour (1HR condition) or 5 minutes between sessions (5Min condition). As in the previous experiments, all rats receiving spaced conditioning were returned to their home cage between sessions. Four days following completion of the conditioning, the rats receive sham or HPC lesions and were tested for retention as described in the previous experiments. Histological analyses of the lesions also followed the procedures described in experiments 1 and 2.

4.2 Results

4.2.1 Histology

The data from 5 HPC rats were excluded from the experiment for not meeting the minimum HPC lesion size criteria (3 from the 1HR group and 2 from the 5Min group) resulting in 6 HPC rats per group. The average HPC lesion size for each group is displayed in Figure 8, and a t-test found no significant difference in HPC lesion size between groups, t (10) = 0.914, p = .1064.

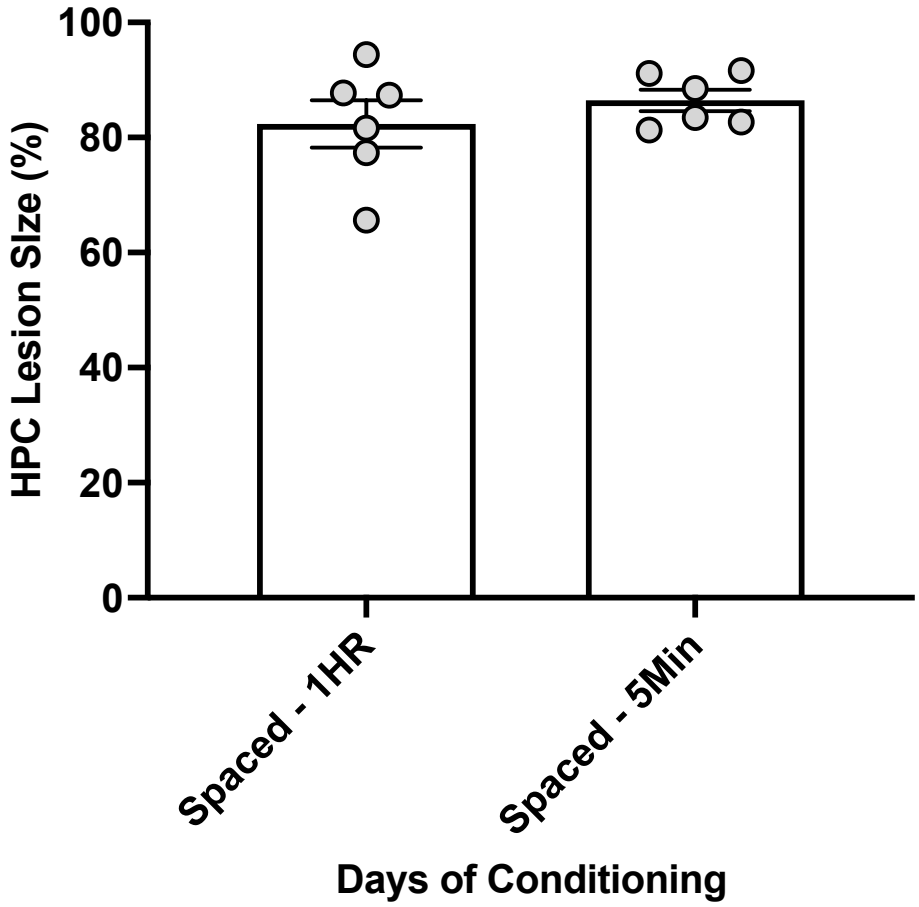

Figure 9. Mean (+/-SEM) lesion % of the HPC lesion rats in both groups. No significant difference was found in lesion size between the groups (*ps* > .05).

4.2.2 Behavioural Results

4.2.2.1 Conditioning

The rats were randomly assigned to receive either Sham or HPC lesion prior to conditioning and data their pre-shock freezing data across acquisition sessions are presented in Appendix A. A mixed design ANOVA with Sessions (1-6) as the within-subjects factor and Surgery (Sham or HPC) and Interval (1HR or 5Min) as between-subjects factors was used to examine possible acquisition differences amongst the groups. The ANOVA revealed a significant Session x Interval interaction F (5, 120) = 10.23, $p < .001$, indicating that the rats from the 1 HR condition showed more freezing across acquisition sessions than the rats in 5 Min condition. No significant Session x Surgery F (5, 120) = 1.65, $p = .153$ and Session x Interval x Surgery F (5, 120) = 0.72, $p = .613$ interactions were significant. Hence, Sham and HPC rats had similar freezing across sessions within their respective conditions. Post hoc analyses of the significant interaction revealed that the rats in the 1HR condition and the 5Min condition differed significantly in freezing behavior only at Session 4 ($p = .009$) and 5 ($p = .006$) of acquisition, where rats in the 1HR condition froze more than those in the 5Min condition. However, there were no significant differences in sessions 1, 2, 3, or 6 (ps > .05). Also, there was a

significant main effect of Session F (5,120) = 99.35, $p < .001$, Interval F (1,24) = 5.32, $p = .03$, but not Surgery F (1, 24) = 1.97, $p = .174$. Because of the significant main effect described above, however, none of these significant main effects were further analyzed.

4.2.2.2 Retention

An ANOVA with Surgery (Sham, HPC) and spaced learning interval (Spaced-1HR, Spaced-5Min) as between-group factors revealed significant main effects of Surgery, F (1, 24) = 18.33, $p < .001$ and spaced learning interval, F (1, 24) = 10.43, $p = .004$. These main effects were not further analyzed because a significant Surgery x Spaced Learning Interval interaction was found, F (1,24) = 7.062, $p = .014$, indicating that the effect of surgery on retention varied with the way learning occurred. Pairwise comparisons using LSD showed that the freezing of the 1HR HPC rats was not significantly different than that of the 1HR Sham rats ($p = .262$), indicating that the HPC lesions did not result in retrograde amnesia for this group, replicating the findings of Experiment 2. However, freezing of the 5Min HPC rats was significantly lower than that of the 5Min Sham rats ($p < .001$), indicating that the HPC lesion resulted in retrograde amnesia and suggesting that 5 minutes between spaced learning sessions is not enough to create a memory independent of the HPC.

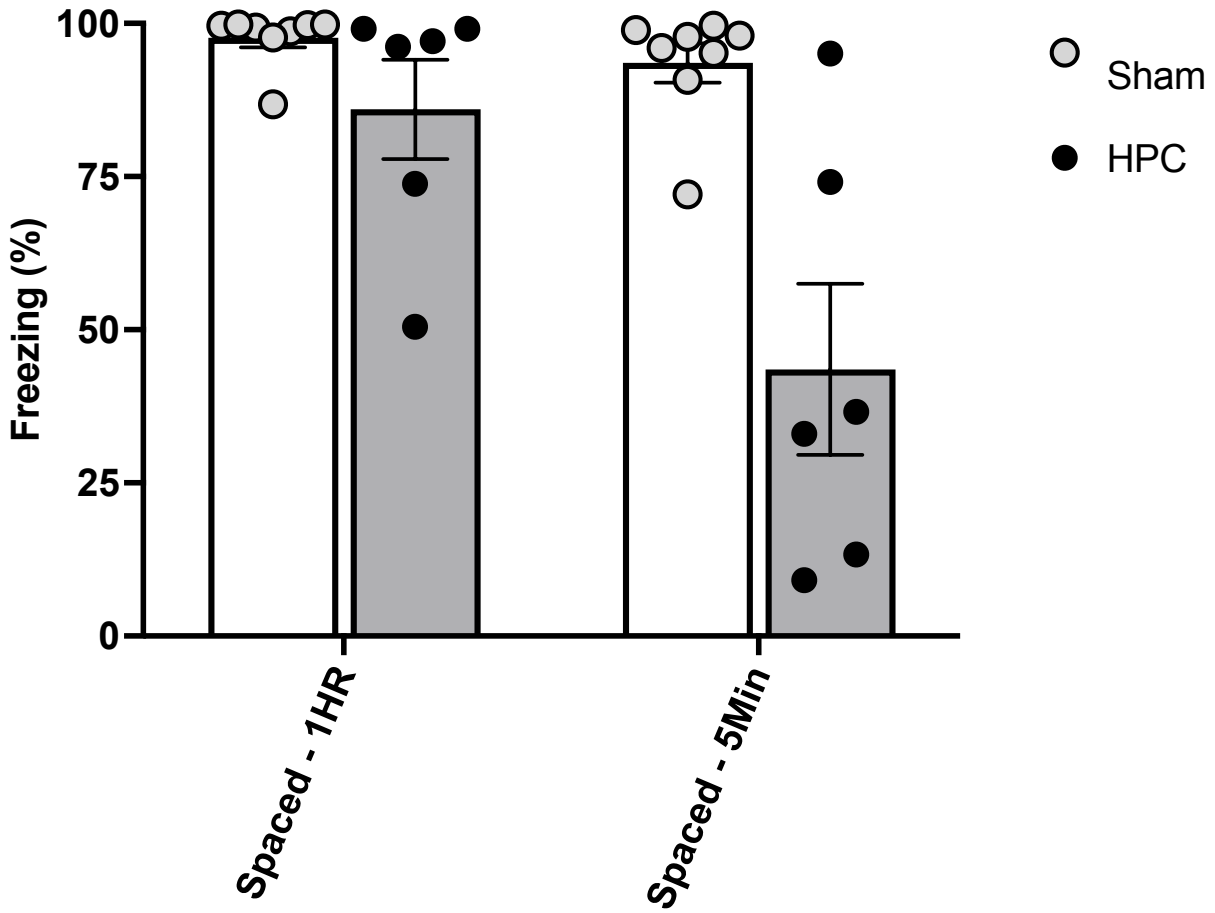

Figure 10. Mean (+SEM) percent time freezing by Sham and HPC rats during the Experiment 3 retention test. The HPC rats in the Spaced - 5Min group froze significantly less than their respective control rats ($p < .001$), suggesting that the HPC damage caused retrograde amnesia in these rats. The freezing of the HPC rats in the Spaced - 1HR group was not significantly different than their control rats ($p > .05$), suggesting that these rats did not suffer from retrograde amnesia.

5. General Discussion

The main objective of this study was to examine whether incremental consolidation bouts resulting from spaced learning sessions facilitate the formation of memories resistant to HPC damage. This was achieved by manipulating the intervals between sessions with opportunity for separate bouts

of cellular consolidation in mind. Experiment 1 demonstrated that six contextual fear conditioning sessions spaced over three days are sufficient to make a memory independent of the HPC. Experiment 2 showed that spacing six conditioning sessions within a single day separated by one-hour intervals, a sufficient temporal window for new separate bouts of cellular consolidation to occur, also produces an HPC-independent memory. Finally, Experiment 3 showed that reducing the interval to five minutes, a temporal window argued to be insufficient for new separate bouts of cellular consolidation to occur, did not produce an HPC-independent memory, ruling out things like context changes that may occur when rats were returned to their home cages between context-shock pairings as possible explanations. Once it was established in Experiment 1 that the minimum spaced context-shock pairings needed to make an HPC-independent memory was 6, all ensuing groups (Massed, 3D, 1Hr, 5Min) spent the same total time in the context and received the same number of context-shock pairings, ruling out cumulative time and shock pairings as the key factors influencing the results (Table 2). Together, though, the results across all three experiments do support the broader postulation that spaced learning strengthens memory traces through incremental cellular consolidation bouts, as demonstrated by the

necessity of both a minimum interval that allows for cellular consolidation bouts, and a minimum number of learning events.

Table 2. Summary of Results Across All Three Experiments.

Group	Experiment (replicated)	HPC n	# of context-shock pairings	Total minutes spent in context during acquisition	Returned to homecage between context-shock pairings?	HPC Lesion %	Retrograde Amnesia?
1D	Exp 1	9	2; 2 per day (AM/PM) for 1 day	6; 2 x 3 minute sessions	Y	87.39	Y, p<.001
2D	Exp 1	9	4; 2 per day (AM/PM) for 2 days	12; 4 x 3 minute sessions	Y	81.14	Y, p<.05
3D	Exp 1 (Exp 2)	9 (5)	6; 2 per day (AM/PM) for 3 days	18; 6 x 3 minute sessions	Y	86.54 (84.15)	N, p=.958 (p=.886)
4D	Exp 1	10	8; 2 per day (AM/PM) for 4 days	24; 8 x 3 minute sessions	Y	84.09	N, p=.967
5D	Exp 1	10	10; 2 per day (AM/PM) for 5 days	30; 10 x 3 minute sessions	Y	91.61	N, p=.482
Massed	Exp 2	9	6; 1 session in 1 day	18; 1 x 18 minute session	N	84.4	Y, p<.001
1HR	Exp 2 (Exp 3)	7 (6)	6; separated by an hour in 1 day	18; 6 x 3 minute sessions	Y	83.65 (82.35)	N, p=.866 (p=.262)
5Min	Exp 3	6	6; separated by five minutes in 1 day	18; 6 x 3 minute sessions	Y	86.46	Y, p<.001

It is important to emphasize that reducing the interval between spaced learning sessions from one hour down to five minutes in Experiment 3, a window thought to be insufficient for new bouts of cellular consolidation, prevented the benefits of spaced learning on the formation of a HPC-independent memory. Indeed, the HPC rats that received their six conditioning sessions spaced five minutes apart suffered from retrograde amnesia whereas those that receive their six spaced sessions one hour apart did not. However, since there was a significant interaction found during acquisition between session an interval, there may have been concern that the retrograde amnesia difference was due to overall poorer

learning in the rats from the 5Min condition. However, this is unlikely the case as pre-shock freezing during the last acquisition session was comparable between the rats in the 5min and 1HR conditions. Moreover, during the retention test, the control rats from the 5Min condition showed comparable freezing levels as the ones form the 1HR condition. Hence, learning and memory was strong in the 5Min condition. The retrograde amnesic effects of the lesions were likely because the memory trace in the non-HPC system did not have opportunity to benefit from as many repeated consolidation bouts and become sufficiently strong in this system to withstand extensive HPC damage.

The DRT introduced by Sutherland et al. (2010) suggests that spaced repetitions of a learning episode can strengthen the memory trace in structures outside the HPC such that the HPC is no longer required to recall the memory. The DRT is supported by the findings of Lehmann et al. (2009), who showed that spaced reinstatements of a CFC learning episode over consecutive days created a memory independent of the HPC, and Guldbrandsen et al. (2013), who found that inactivating the HPC during retention testing following spaced CFC learning did not result in retrograde amnesia, given a sufficient number of spaced learning sessions. The DRT, thus, provides a framework for understanding how spaced learning strengthens a memory trace outside the HPC. The DRT would suggest

that each repetition or reinstatement induces a new bout of cellular consolidation, incrementally strengthening the memory trace in non-HPC systems, and therefore it is the spaced repetitions that are the mechanism by which the transition from HPC-dependent to HPC-independent memory occurs, rather than simply the passage of time (Sutherland et al., 2010; Lehmann and McNamara 2011). In short, the DRT posits that each repetition or reinstatement incrementally strengthens the memory trace, induces a new cellular consolidation bout, and ultimately establishes the memory trace outside the HPC.

Our findings lend support to each of the three components mentioned in the DRT. First, we find support for the DRT's posited incremental effect of spaced learning in Experiment 1, which shows a progression in memory retention until asymptote is reached after three days and six learning sessions (Figure 4). The rats that received two days and four learning sessions displayed higher retention than those that received one day and two sessions, but lower than those that received three days. This pattern suggests an incremental effect of the learning sessions. Second, in further support for the DRT, the memory sparing observed following HPC damage aligns with the DRT's assertion that the memory trace strengthened through spaced learning becomes supported by non-HPC structures. Consistent with this view, Shepherd et al. (2021) reported that context memories established

through spaced learning survive damage to the HPC only as long as the perirhinal cortex and anterior cingulate cortex remain intact. Hence, the perirhinal cortex and anterior cingulate cortex are critical structures within the non-HPC memory system that supports a memory transitioning to HPC-independence via spaced learning.

Third, we observed retrograde amnesia when the intervals between spaced learning sessions restricted the opportunity for new bouts of cellular consolidation, but no retrograde amnesia when there was opportunity for cellular consolidation. By manipulating these opportunities in a naturalistic manner rather than via pharmacological interventions, we were able to determine the temporal parameters necessary to create an HPC-independent memory, account for the context changes between conditioning sessions and rule out any role they may play in the transition of a memory from HPC-dependent to HPC-independent. It also eliminates the need to differentiate between consolidation and reconsolidation, which may overlap and rely on different specific cellular processes that would need to be accounted for in a pharmacological intervention (Bozon et al. 2003; Lee, 2008; Lee and Hynds, 2013), as this distinction was not necessary to achieve the present objective. Future use of pharmacological interventions such as those that temporarily inactivate brain regions, or those

that inhibit or promote protein synthesis, the key mechanism of cellular consolidation, will further test and refine the parameters identified in our findings. For instance, utilizing a design of two spaced CFC sessions, Lee (2008) found that initial consolidation (which occurs in the first session) requires BDNF but not Zif268, whereas reconsolidation (which occurs in the second session) requires Zif268 but not BDNF. Thus, future use of pharmacological interventions could facilitate an exploration and expansion of Lee's (2008) findings within the findings of the present study. For instance, progressively expanding Lee's (2008) methodology beyond their two spaced sessions until reaching our established minimum of six would allow further examination of consolidation and reconsolidation, and the place of BDNF, Zif268, and other protein synthesis mechanisms may have beyond the initial two spaced sessions.

Theories explaining the memory benefits of spaced learning often fall under one of four overarching categories: deficient processing theories, encoding variability theories, study-phase retrieval theories, and consolidation theories, or some hybrid thereof. Of particular relevance to this study, study-phase retrieval theories propose that repetition of the learning trials reactivates the earlier representation, which strengthens it in long term-memory (Thios and D'Agostino 1976; Hintzman and Block 1973; Hintzman, 2004; Benjamin and Tullis, 2010), and

consolidation theories of spaced learning propose that in order to see the benefits of spaced learning there must be a sufficient window between trials to allow for cellular consolidation to occur (Landauer 1969; Wicklegren 1972; Toppino and Gerbier, 2014; Gerbier and Toppino, 2015; Smolen et al., 2016). Consolidation theories of spaced learning are not necessarily mutually exclusive with the other theories; in fact, there are suggestions of hybrid theories, or that consolidation could underlie some of the findings traditionally used to support the other theories (Delaney, Verkoeijen, & Spirgel, 2010). Indeed, a hybrid cellular consolidation / study-phase retrieval theory of spaced learning would share similarities with Sutherland et al.'s (2010) DRT.

There was, however, interesting individual variation among the rats that received two days of spaced learning, with some exhibiting clear retrograde amnesia and others exhibiting none. This may suggest that varying individual memory thresholds for each rat were being reached (or not reached) during training. It is worth noting, however, that in experiment 1 the mean lesion size for the rats that received their spaced learning over two days (81.14%) was significantly smaller ($p < .05$) than the rats that received their two spaced learning sessions over one day (87.39%). We did not further examine this because the HPC lesions were still of sufficient size to induce retrograde amnesia (Scott et al.,

2016), and once maximum retention was observed at three days it established our minimum number of sessions/days to create an HPC-independent memory via spaced learning. The variation in retention among the two-day rats, as well as the difference in lesion size between the one and two day groups, however, means we cannot fully rule out some manner of memory threshold being at play in the transition of a memory from HPC-dependent to independent (e.g., perhaps the memory trace can become HPC-independent after four spaced context shock pairings but the group threshold is reliably reached after six, or perhaps with equal lesion size the pattern would not appear incremental).

The findings of the present study have implications for how we think about temporally graded retrograde amnesia (TGRA), a phenomenon famously reported by Scoville and Milner (1957) in their study of patient H.M., whereby individuals with HPC damage can recall remote memories from long before their injury but struggle to recall recent memories formed shortly before the damage. Specifically, Scoville and Milner (1957) observed that, following a medial temporal lobectomy, patient H.M. suffered both anterograde and retrograde amnesia, and that H.M.'s retrograde amnesia was temporally-graded; he could recall remote declarative memories from childhood, but not recent declarative memories from the years leading up to his lobectomy. A few studies report similar TGRA in rats that

received HPC lesions following CFC (see Kim and Fanselow, 1992; Anagnostaras et al., 1999). However, many other studies examining the effects of HPC lesions have failed to find evidence of TGRA in CFC (Lehmann et al., 2007; Sutherland et al., 2008; Sutherland et al., 2010; Sparks et al., 2011). Regardless, TGRA has traditionally been taken as key evidence supporting systems consolidation theories (Squire, 1992; Nadel and Moscovitch, 1997; Winocur et al., 2013), which posit that memories initially rely on the HPC but gradually become independent of it over an extended passage of time as they are strengthened in other brain areas. However, our findings provide an alternate explanation, showing that repeated, spaced events that engage multiple cellular consolidation bouts can make a CFC memory independent of the HPC, and that this can occur within a short period of time.

In conclusion, spaced learning, even within a single day, can lead to the formation of a memory that is independent of the HPC provided there is sufficient opportunity for multiple cellular consolidation bouts to strengthen the memory in areas outside the HPC.

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Appendix

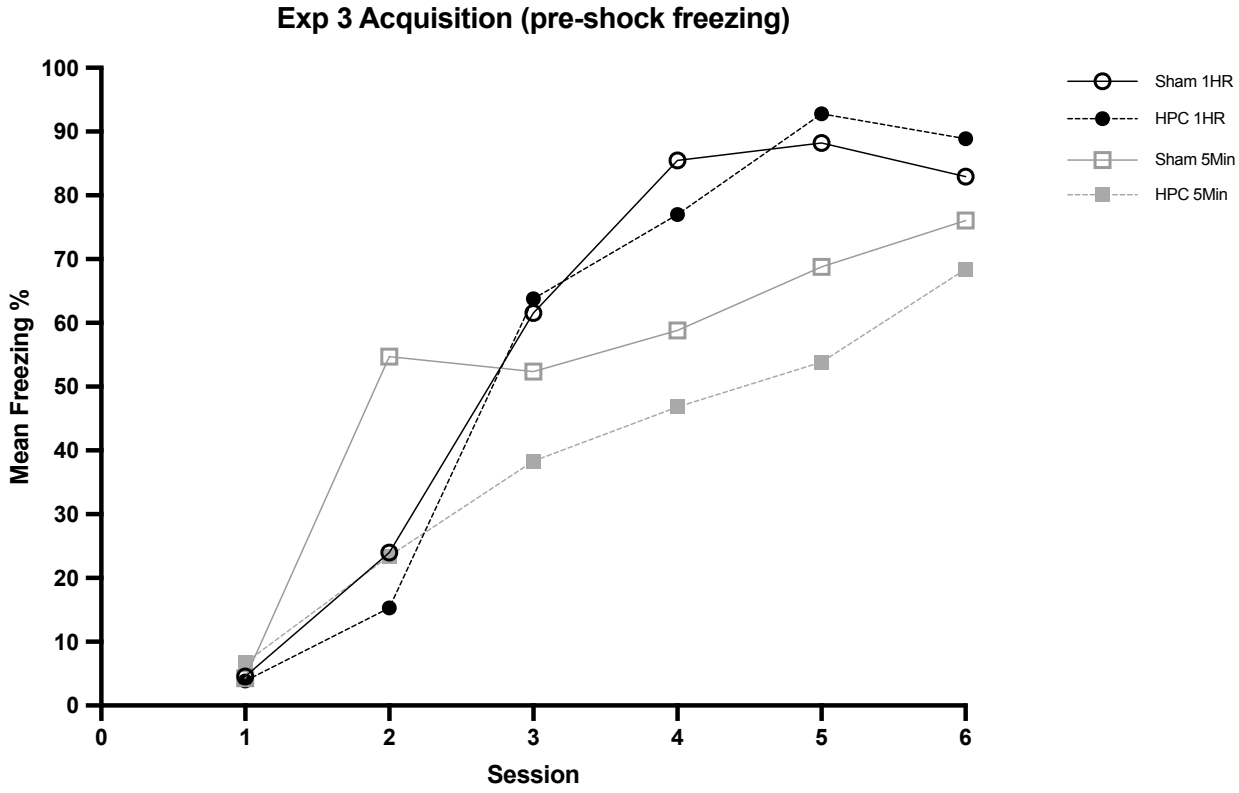

Figure 11. Acquisition data of rats in Experiment 3. Rats received spaced conditioning of 6 single-shock sessions in one day, separated by either an hour (1HR condition) or 5 minutes between sessions (5Min condition). Rats then received either Sham or HPC lesion surgery; thus *Sham* and *HPC* here denotes the eventual surgery these rats received following acquisition, but they had not yet been surgerized at the time of acquisition.