CONTEXT FEAR MEMORY: ESCAPING THE HIPPOCAMPUS

A Thesis Submitted to the Committee on Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Faculty of Arts and Science

TRENT UNIVERSITY
Peterborough, Ontario, Canada

Psychology M.Sc. Graduate Program
May 2019

ABSTRACT

Context-fear memory: Escaping the Hippocampus

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Distributing contextual fear episodes makes the memory become HPC-independent, meaning increasingly reliant on non-HPC memory structures. It is unclear, however, whether distribution of the conditioning episodes alone is sufficient or whether a combination of distribution and high conditioning saliency is necessary to make the memory become HPC-independent. To resolve this issue, rats were trained using a distributed contextual fear conditioning protocol in which foot-shocks were manipulated to create a low (0.4mA), intermediate (0.7 mA) and high (1.0 mA) saliency condition. This thesis also aimed to determine brain structures supporting the HPC-independent memory by assessing retention-induced c-fos expression in the basolateral-amygdala, perirhinal and anterior cingulate cortices. The results suggest that HPC lesion rats in the high saliency condition displayed similar level of freezing as control rats, indicating "strongly salient" and distributed episodes creates a HPC independent memory. c-fos expression suggests together, an increased context representation in the perirhinal and anterior cingulate cortices and a strengthened fear representation in the basolateral-amygdala supports the HPC-independent memory.

Keywords: memory, retrograde amnesia, hippocampus, context fear, amygdala, anterior cingulate cortex, perirhinal cortex, consolidation, saliency, reinstatements

ACKNOWLEDGEMENTS

A heartfelt thank you to my supervisor Dr. Hugo Lehmann, for his guidance, wisdom and teaching throughout my study at Trent University. You contributed significantly to my interest and love for neuroscience. You were always there to listen and offer insight to problems I encountered along the way, and for that I am so thankful. I couldn't have wished for a better teacher.

Additionally, I am grateful to all the wonderful professors at Trent University for making my experience one I would never forget. Also, I am thankful for my lab mates especially Elizabeth Shepherd, Sydney MacLeod, Chloe Carter, Kelsey Jungkunz and Jenna Mackenzie for their kindness, help and advice in completing this thesis.

Finally, I would like to say thank you to my husband, Vishol Kishun, for all his help and support. Your words of encouragement pushed me when I was not motivated to continue, so thank you! Also, thank you mom for always believing in me. To my darling daughter, Vitalya Kishun: your love and laughter filled my heart with enough inspiration to see me through.

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Chapter 1-Introduction

Smothered in a crowded NYC train headed to the Upper East Side Manhattan Neurology office where I work, I busted into the office angrily. I was angry because there were major delays in the train service, so I had to stand an extra 20 minutes smelling the armpit of the guy standing beside me. I was a bit more ticked off to enter the office and find the first patient already sitting in the waiting room, meaning I had no chance of getting coffee before I attempted to salvage the day. It was Mr. Bob, a wealthy man and former White House official. He is a regular patient, but I had not seen him in the office for a while. In my opinion he is a stand-up man who does not talk much. He noticed that I was upset, so he said, "Here, come sit", offering me a seat next to him. Despite the rush I was in, I did not want to seem rude, so I sat. Mr. Bob asked, "do you know what the word *shit* means?" Surprised by his question, I said, "yes, it means *shit*... you know people use it to mean all sorts of things, like to express surprise, anger, happiness, it's a vulgar word..." He replied, "No, no, no, let me tell you what the word *shit* means." He went on to explain that "back in the day" when ships were used to transport manure from one place to another for farming, ship operators often stored manure in the lower decks of the ships and because of the buildup of methane gas, there would be explosions on the ships. After learning that it was the methane gas that caused the explosions, operators decided to store the manure high in transit. Instead of writing "store high in transit" on every manure bag they wrote s.h.i.t for short. I laughed! I learned something fascinating! Something I can talk about with my friends the next time I am at a party! Mr. Bob's story lightened my mood.

Mr. Bob is a patient in the office because he suffers from amnesia following a car crash that gave him a traumatic brain injury. Importantly, the permanent brain damage includes, at least in part, a brain area critical for memory called the hippocampus (HPC). This structure is

believed to be critical in storing declarative memories, memories you can consciously recall, memories like Mr. Bob's memory of this story. There are two types of declarative memories, namely episodic and semantic memories, which I will elaborate on later in the thesis. But, as I think about an intriguing story to capture my reader's attention as I attempt to write this thesis, I recalled Mr. Bob's story and had to retell it. It was only trying to write the story now, that I decided to research whether the story was true or whether it was a false etymology. It turns out, Mr. Bob was actually full of *shit*! The story is not true at all, so I beg, please don't retell it! He learned of the *shit* epidemiology from a 2006 film called *Kenney* he saw with his late wife- *I asked!*

Mr. Bob does not remember much of his past, but he vividly remembers the *shit* story and he tells it passionately. This declarative and rich in detail memory, however, should require an intact and properly functioning HPC (Nadel & Moscovitch, 1997), but not in him. Why? This thesis focuses on this issue or how HPC dependent-memories (memories that need the HPC to be expressed) may become HPC-independent (no longer requiring the HPC to be expressed). Moreover, this thesis aim in providing insight on where the memories go, meaning what structures come to support the HPC-independent memories. So, before we delve further into why some memories are better remembered than others and how we could make a memory that was initially dependent on the HPC, independent of it, let us consider the general anatomy about the HPC and some history about the functional role of the HPC. Following this, I will review current and emerging views about the role of the HPC in supporting memory.

1.1 The Hippocampus

1.1.1 Brief Historical overview and Anatomy

In the dawn of 16th century Caesar Arantius who assisted his surgeon uncle with anatomical dissections, discovered the HPC (Bir, Ambekar, Kukreja, & Nanda, 2015). When he dissected the brain from the ventral surface, he noticed that a structure in the medial temporal lobe looked like a seahorse, so he gave the it the name "hippokampus" meaning "sea horse" (Bir et al., 2015). Following that, researchers were caught by the elegant curved features of the HPC and named them cornu ammonis (CA), Latin for horn of the ram because the curves resembled the horn of a ram. The sea-horse looking structure consisted of another part that was not the CA fields. This structure is the dentate gyrus, which received its name because it looked like a series of bumps on the cerebral surface running along the length of the gyrus. The row of bumps resembled a row of teeth, hence the structure was given the name dentate (teeth-like) gyrus (Mark, Daniels, Naidich, Yetkin, & Borne, 1993). The HPC now is typically defined anatomically as the CA fields (CA1, CA2, CA3 and CA4) as well as the dentate gyrus (Bir et al., 2015; Dejong, 1973).

When considering the HPC and its connections with other structures, the HPC is viewed as the structure in which sensory information gets sorted and processed. Information enters the HPC through three main pathways: the perforant, the mossy fiber, and the Schaffer collateral pathways. The perforant pathway originates in the entorhinal cortex and terminates in the dentate gyrus, while the mossy fiber pathway starts at the dentate gyrus and end at the CA3 region. The Schaffer collateral pathway originates at the CA3 region of the HPC and ends at the CA1 region. Information enters the HPC from most of the cortex and subcortical regions either directly or indirectly via the entorhinal cortex(Kubik, Miyashita, & Guzowski, 2007). Outputs from the

HPC pass through the fornix to the anterior nucleus of the thalamus, then to the cingulum, subsequently to the entorhinal cortex and then back to the HPC. This looping is commonly referred to as the Papez circuit (Papez, 1937).

During the 18th century, the HPC was viewed as playing an important role in a number of different functions, including smell, taste olfaction and attention. In 1876, Sir David Ferrier suggested the sense of smell was localized in the temporal lobe. He conducted research on monkeys and reported that damage to the temporal lobe including the HPC resulted in a smelling deficit. He also reported that with damage to the HPC "[the monkeys] they had lost the faculty of attentive and intelligent observation." He also believed that tactile sensation was impaired in proportion to the amount of damage caused to the HPC (Ferrier, 1876). Soon after, in 1928 Horsley introduced an opposing view. He found that extensive lesion of the HPC did not lead to impairment in tactile stimulation. Furthermore, shortly after Brown (1890) reported that when he operated on monkeys, thereby removing most of their temporal lobes, the animals gave distinctive indications of still possessing smell and taste.

Many of Ferrier's experiments were not supported by studies conducted after the 18th century, but he introduced a ground-breaking way of thinking. He was a philosopher who did not philosophize but who experimented. He was also influential in initiating brain surgery as he encouraged surgeons to operate on the human brain to understand the contribution of various structures to behaviour. Below I will discuss advancements in the study of the HPC following the era of Sir David Ferrier's experiments.

1.1.2 Localizing memory and following the seahorse

Over a century ago Karl Lashley began exploring whether there are brain structures responsible for specific functions (see Lashley, 1950). He began looking for the engram, meaning the group of brain cells where the physical representation of a memory may reside. He trained rats to navigate a maze then lesioned part of their brain to see whether that would lead to memory impairments. He was using the lesions to try and erase the physical representation of the maze memory in the rats, but he found that he was unable to do so. Nevertheless, he found an interesting pattern (Lashley, 1950). Localized damage did not result in amnesia but increased damage, regardless of location resulted in memory deficits, a phenomenon became known as mass action. He went on to explain that memory is widely distributed in the cortex and not localized in one structure. Lashley was unable to locate an engram, but research since then has revealed that memory can be localized, at least in part, to specific structures.

1.2 The HPC as a Memory Center

Some evidence suggesting supporting localization of function and memory came from Penfield electrical stimulation of the neocortex. Wilder Penfield demonstrated that electrical stimulation of the brain, particularly the temporal lobe and the HPC, could elicit memory and perception (Penfield & Boldrey, 1937). Penfield applied small jolts of electricity to the brain with the intension of revealing regions responsible for causing seizures but instead found that stimulating parts of the temporal lobe caused patients to have vivid recall of episodic memories (Penfield & Boldrey, 1937). For example, one patient vividly recalled a memory with her friends in South Africa. She exclaimed, “Yes Doctor, yes, Doctor! Now I hear people laughing- my

friends in South Africa." The most compelling evidence however came from a case study of Patient H.M who will be elaborated on in the next section.

1.2.1 The Case of H.M. and other Patients with HPC Damage

One of the earliest studies that led to the discovery that the HPC plays a critical role in memory was the case study of Patient H.M. He had sustained a head injury at age 9 that eventually led to epilepsy (Scoville & Milner, 1957). To alleviate his seizures, part of his temporal lobe, including a large part of his HPC, was removed. The surgery was effective in mitigating the seizures, but resulted in several amnesic side effects. Following the surgery, H.M started suffering from retrograde and anterograde amnesia. More specifically, he had amnesia for declarative memories, the kind you can consciously bring to mind. Declarative memory is divided into two sub-groups namely, semantic and episodic memories. H.M had amnesia for episodic memory such as conscious recall of specific life events, including details about when the events took place and where the information was acquired. His semantic memory, meaning his recollection for general knowledge and facts was unaffected. Moreover, his procedural memory, such as skills and habits, was unaffected. H.M.'s short term memory remained intact, but his long-term memory was impaired. Clearly, not all of H.M.'s memory functions were affected by damage caused by his surgery. H.M.'s amnesia for declarative memories suggests that the HPC specifically supports such memories. This provides evidence supporting localization of function and provides support for the HPC as a memory center.

More important for this thesis, H.M.'s retrograde amnesia had an interesting pattern: he had loss of memory for recent but not remote events. This pattern of amnesia is known as temporally graded retrograde amnesia (TGRA). Memories acquired just before H.M.'s surgery, such as the death of his favorite uncle that occurred three years prior to his surgery, were lost but

other childhood events, such as a teenage relationship he had, remained intact (Scoville & Milner, 1957; Squire & Wixted, 2011). H.M. also recalled faces of persons who became famous in his early years, but not faces of persons that were in the news leading up to his surgery (Squire, 2009).The findings of TGRA are suggestive that the temporal lobe including the HPC plays a time-limited role in memory, whereby memories are "transferred" from the HPC to neocortical structures over an extended period of time, a process termed long-term systems consolidation.

Several other case studies reported this same pattern of memory loss including patients R.B and E.P, who had more localized HPC damage. Patient R.B, a 52 year old postal worker suffered a stroke that resulted in neural damage restricted to the CA1 region of his HPC (Rempel-Clower, Zola, Squire, & Amaral, 1996). Like patient H.M., he also had TGRA and could not remember events that happened approximately two years prior to his stroke. He could not recall ever having had a stroke or being in the hospital. On one experimental test, R.B was asked to recognize 74 television programs that broadcasted for a single season between 1963 and 1977. He suffered his stroke in 1978 and showed memory deficits only for programs aired between 1975 and 1977. It is fascinating that he was able to successfully recall the programs that aired before 1974 as well as control participants. Thus, he had intact remote but impaired recent memory (Zola-Morgan, Squire, & Amaral, 1986). Another case is that of Patient E.P who became amnesic at age 70 when viral encephalitis destroyed his medial temporal lobe (Squire, And, & Knowltont, 1995; Stefanacci, Buffalo, Schmolck, & Squire, 2000). Most notably, E.P had extensive bilateral HPC damage and also presented with TGRA. He was impaired on tests of recall and recognition for public events, famous faces and famous names that came into the news years before his surgery but scored average or better than age matched controls for subject

matters that were in the news during his early childhood (Reed & Squire, 1998; Stefanacci et al., 2000). Combined, these cases suggest that the HPC plays an important, but transient, role in memory. The HPC would be involved in supporting memories for a period, in these cases a few years, until they are "transferred" to other structures more permanently. Basically, these cases suggest that some memories can initially be HPC-dependent, meaning require support from the HPC, but over a protracted period become HPC-independent, meaning that they no longer critically require the HPC for expression. Although these cases suggest that the HPC plays a transient role in supporting memory, there are other cases of patients with HPC damage suggesting that the HPC is permanently involved in memory (Crokin, 2005;Rempel-Clower et al., 1996; Maguire, Nannery, & Spiers, 2006 & Zola-Morgan et al., 1986), but this evidence will be described and discussed in a later section.

1.2.2 HPC Damage in Non-Human Animals

Overall, in the field, there is an agreement that HPC damage in non-human animals results in retrograde amnesia on multiple memory tasks, ranging from those involving simple forms of conditioning to those involving more complex associations amongst several events or features (configural memories) (Epp et al., 2008; Martin, De Hoz, & Morris, 2005; Sutherland, Brien, & Lehmann, 2008; Sutherland, Sparks, & Lehmann, 2010). There are many advantages to using non-human animals when studying the role of the HPC in memory. Non-human animals allow the researcher to control the exact time of the learning episodes, strength of memory, type of memory assessed, extent of HPC damage, the nature of intervening experiences between learning, time of targeted brain damage or inactivation, etc. (Sutherland et al., 2010). I will now discuss instances in which HPC lesions caused TGRA in non-human animals similar to what had been observed in patients with HPC damage.

Brown and Schafer (1887) were the first to report memory impairment in non-human animal following HPC lesions. Specifically, they lesioned large parts of the temporal lobe, including the HPC, in rhesus monkeys and found that following the damage the monkeys investigated objects they had previously interacted with, as if they were entirely new and unknown (Brown & Schafer, 1887). Reports of TGRA for object memory, however, were only reported much later (Cho, Beracochea, & Jaffard, 1993; Zola-Morgan & Squire, 1990). Zola-Morgan and Squire (1990) trained monkeys to discriminate 100 pairs of objects beginning 16, 12, 8, 4 and 2 weeks before the hippocampal damage. Monkeys with HPC damage were severely impaired in recognizing objects learned recently (2 week) but not remotely (12 weeks). In this instance, the monkeys displayed TGRA for an object discrimination task. Even in a non-human animal, like the monkey, the HPC plays a seemingly temporary role.

A large portion of what we know about the role of the HPC in memory comes from studies conducted on rodents. In rodents, HPC damage results in memory deficit on spatial and object-recognition, trace eye-blink, flavor/odor, socially acquired food preference and contextual fear memories tasks (Anagnostaras, Maren, & Fanselow, 1999; Clark, Broadbent, & Squire, 2007; Epp et al., 2008; Kim & Fanselow, 1992; Tse et al., 2007; Winocur & Moscovitch, 1990). The first report of TGRA in rodent was produced by Winocur et al. (1990). He trained rats using an acquired food preference protocol in which a naive rat was paired with a demonstrator rat that recently sampled a distinctively flavored food. Through interaction with the demonstrator rat, the subject rat acquires a preference for that food. The amount of food consumed was used as a measure of the rat's preference. The rats were lesioned 1, 2 ,5 or 10 days after they interacted with the demonstrator rats and acquired the food preference. They reported that the HPC lesioned rats consumed significantly more of the sample food during the retention test at the

remote time point (10 days between acquisition and surgery) when compared to the recent (e.g. 1 day). They further found that even with complete lesion of the HPC, TGRA for a socially acquired food preference was still evident.

Shortly after, Kim and Fanselow (1992) demonstrated that the HPC plays a time-limited role in associative contextual fear memories, but not tone sensory stimuli. They trained rats using tone-foot shock pairings in a distinctive chamber. Following training, bilateral lesions were made to the HPC either 1, 7, 14 or 28 days later. They were then tested for retention where they were introduced back into the context without the presentation of the foot shock or tone. HPC-lesioned rats showed significantly higher level of freezing at the remote (28 day) time point compared to the recent (1 day) condition, thereby suggesting TGRA. On the next day, the animals were placed in a different chamber and were presented with the conditional tone. Fear memory of the tone was assessed by freezing percentage. They reported that rats did not freeze in the new chamber, suggesting that the context fear conditioning was specific and did not generalize to the new chamber. Also, the HPC lesion did not affect retention of the tone at any time points therefore, lesion did not affect the tone-elicited freezing. They went on to explain that the HPC is important in the retention of context-shock memories but not fear memory based on a tone-shock association.

TGRA in contextual fear conditioning (CFC) was even found using a clever within-subject design. Anagnostaras et al. (1999) illustrated that partial HPC lesion resulted in memory deficit for recently but not remotely acquired context fear memories (Anagnostaras et al., 1999). Rats were given 10 tone-shock pairings in one context, 50 days later, they received another 10 tone-shock pairing in another context with a different tone. A day after the second learning event they received either HPC or sham surgery. They were then tested for retention of the remote

context and then the recent context the day after. HPC-lesioned rats displayed equivalent levels of freezing (used as index of memory) as sham animals to the remotely acquired context. The same rats displayed severe amnesia of contextual memory that was 1 day old at the time of lesion. They concluded, again, that the HPC plays a role in memory storage that is time-limited. As more time passes, the HPC passes the memory to other brain structures and it is therefore not necessary for the expression of the memory.

As of yet, the described studies suggest that damage to the HPC in humans and non-human animals can cause TGRA. These studies lead to the conclusion that the HPC plays a temporary role in supporting memories, one that can extend weeks or years, but not permanently (Anagnostaras et al., 1999; Kim & Fanselow, 1992; Takehara, Kawahara, & Kirino, 2003). Again, it is important to emphasize that it often reported that HPC damage fails to cause TGRA and that the memory loss in these cases is equivalent for recent and remote memories (see Sutherland et al. 2010 for review) and this will be discussed in section (1.4). However, prior to that, the next section presents evidence from functional imaging studies that seemingly support the view that the HPC is transiently involved in memory.

1.2.3 Evidence from Imaging Studies

Assessing memory impairments in patients with HPC damage or in non-human animals with HPC lesion is one way of looking at the role of the HPC. Another way to examine activation of the HPC during recent and remote memory recall using imaging techniques. Several functional magnetic resonance imaging (fMRI) studies have outlined HPC memory systems that are involved in learning and memory (Chadwick, Hassabis, Weiskopf, & Maguire, 2010; Eldridge, Knowlton, Furmanski, Bookheimer, & Engel, 2000; Greenberg et al., 2005; Milner, Corkin, &

Teuber, 1968; Rempel-Clower et al., 1996; Salmon, Bruce, Nelson, & Beatty, 1988; Scoville & Milner, 1957; Squire, Haist, & Shimamura, 1989; Viard et al., 2007). For example, Greenberg et al. 2005 conducted a study in which participants were asked to come up with cue words for 50 autobiographical memories. During fMRI they were asked to press a button when they retrieved the associative memory, that is, when they made the association with the cue word and the autobiographical memory (Greenberg et al., 2005). Greenberg et al. (2005) found that the HPC was activated during episodic memory retrieval for recent memories. They also noted that activation of the HPC during autobiographical memory was greater than the activation during semantic memory retrieval task. They concluded that the HPC plays a greater role in the retrieval of recent episodic memories. Here again, suggesting a temporary role of the HPC.

In another study, Niki and Lou (2002) examined the role of the HPC in storing recent and remote memories by asking participants to recall places they visited 7 years ago (remote) or within the last 2 years (recent). fMRI was done as participants recalled memories from the different time points. Similar to the Greenberg et al. (2005) findings, the researchers reported that there was significantly more HPC activity during the retrieval of recent memories. They further emphasized that their study provided direct evidence of the time limited role of the HPC in forming long term memory (Niki & Luo, 2002). Rekkas and constable (2005) reported the same pattern when they examined the role of the HPC in autobiographic retrieval using a set of questions that probed actual life events. More specifically, they asked participants questions like "can you recall a specific high school English teacher?" or "can you recall the schoolyard of your elementary school?" These questions were used to facilitate retrieval of memories. Rekkas and Constable reported a temporal gradient in activation where they saw more HPC activation in the retrieval of recent memories. All these fMRI studies suggests that the HPC is more activated

during the retrieval of recent memories, thereby signifying that the HPC is more involved in recent than remote memory retrieval (Greenberg et al., 2005; Niki & Luo, 2002; Rekkas & Constable, 2005).

In rodents, similar brain activity evidence following memory retrieval can be found by assessing immediate early gene (IEG) expression. IEGs are a class of genes that are rapidly up regulated following neuronal stimulation caused by a behavioral experience (Brown, Ye, Bronson, Dikkes, & Greenberg, 1996; Farivar, Zangenehpour, & Chaudhuri, 2004). Tasks that require an intact HPC cause increases in IEG expression, including zif268, c-fos and Arc (Guzowski, Setlow, Wagner, & McGaugh, 2001; Guzowski, McNaughton, Barnes, & Worley, 1999; Hall, Thomas, & Everitt, 2001; Vann, Brown, Erichsen, & Aggleton, 2000; Vazdarjanova, McNaughton, Barnes, Worley, & Guzowski, 2002). Thus, assessing IEG expression in the rodent HPC after recall of a recent or remote memory can provide insight in whether the structure is involved in supporting one memory more than the other.

Bruno Bontempi, Laurent-Demir, Destrade & Jaffard (1999), found increased activation of zif268 in a spatial navigation task, specifically the eight-arm radial maze. Rats were allowed to learn that 3 arms of the maze constantly contained food while the other 5 arms never do. Discrimination performance was measured by the amount of correct responses recorded for the first 3 arm choices. Expression of zif268 was then assessed following retrieval at either a recent or remote time point. They reported that retrieval of recent spatial memories produced more robust HPC activation than remote memories, whereas the opposite was found in neocortical areas. Thus, again, support was found for a time-limited role of the HPC in memory (Bontempi et al., 1999).

Hall et al. (2001) found similar results using contextual fear conditioning. They reported an increased activation of Zif268 when rats were tested for retrieval of a recent but not remote contextual fear memory. Rats were conditioned to associate a cue or a context with a foot-shock. Either 24 hr or 28 days after training, rats were returned to the conditioning chamber for a retention test and were subsequently euthanized. Both groups remembered the fear conditioning, but the rats in the 24 hr condition showed higher levels of zif268 activation in the HPC when compared to those in the 28 days condition. They went on to explain that this time-limited activation of zif268 expression in the HPC is consistent with results of animal and human studies demonstrating a temporal gradient (Hall et al., 2001).

1.3 System Consolidation Theories

The studies that report TGRA after HPC damage or the imaging studies suggesting decreased contribution of the HPC when recalling remote memories have had major theoretical implications. They all lead to the conclusion that the HPC plays a temporary role in memory, but also the postulation that the HPC is involved in long-term systems consolidation. The role of the HPC in systems consolidation, meaning a "transfer" of the memory from the HPC to the neocortex over a protracted period of time. The role of the HPC in systems consolidation is not viewed by all in the same way. Actually, there are currently two dominating theories, the Standard Model of Systems Consolidation (SMSC) and the Multiple Trace Theory (MTT), which I will now describe and contrast.

1.3.1 Standard Model of Systems Consolidation

The SMSC suggests that declarative memories usually need the HPC to be expressed, however, if the memory is given enough time, it consolidates in other brain structures. Once

consolidated, the HPC would no longer be required for expression (Marr, 1971). The theory posits that declarative information is encoded in the HPC and non-HPC networks simultaneously and with time, there is a strengthening of the memory in the non-HPC networks. Eventually, allowing the memories to become independent of the HPC, meaning that they can be expressed without it. Importantly, a memory that is consolidated outside the HPC is as rich in detail as the one that was dependent on the HPC. Indeed, HPC-dependent and independent memories are qualitatively the same in this model.

There are important amnesic cases following HPC damage (Corkin, 2002; Warrington & Duchen, 1992) however, that cannot be explained by the SMSC. For instance, some studies report equivalent forgetting for both recent and remote autobiographical memories, where remote episodic memories remain dependent on the HPC and are not consolidated in non-HPC networks (Barr et al., 1990; Warrington & Duchen, 1992, Corkin, 2005).Interestingly, these patients that show retrograde amnesia for episodic memory show TGRA of semantic memories (Barr et al., 1990; Warrington &Duchen, 1992).Thus, HPC-independent memories might be qualitatively different and violate assumptions of the SMSC. In response to this shortcoming of the SMSC Nadel and Moscovitch (1997) proposed the Multiple Trace Theory with a slightly different account of how memories become independent of the HPC.

1.3.2 The Multiple Trace Theory

Nadel and Moscovitch (1997) proposed the MTT which suggests that episodic memories always depend on the HPC, but that these episodic memories also create, over time, a gist-like representation (semantic memory) in non-HPC structures (likely neocortical structures). The MTT emphasizes that a memory, over time, creates an increased number of traces in non-HPC

structures (Nadel & Moscovitch, 1997). These traces are established by speculative "online" as well as "offline" reactivations processes and the increase in the number of traces in the HPC support the episodic information, whereas the traces created in the non-HPC structures are factual representations (gist; semantic) from the original episode. Thus, according to this model, a long-term consolidation process occurs in the HPC for the details pertaining to an episode, whereas a long-term systems consolidation process lead to a gist-like memory outside the HPC (e.g., neocortex). Importantly, the MTT can account for TGRA as well as some flat gradients following HPC damage (Nadel & Moscovitch, 1997).

TGRA for episodic and autobiographical memories can result when there is incomplete HPC damage, because with time the spared HPC tissue would have had enough representation of the episode to support the intact memory. Each reactivation of the memory lays down a new and/or bigger trace in the HPC, such that remote memories are likely to have a greater number and/or more widely distributed traces within the HPC than recent memories. Therefore, with partial HPC damage there is a higher probability that a sufficient portion of the traces for remote memories will be spared to support retrieval. On the other hand, complete HPC damage will always result in a flat-gradient of episodic information, but could also produce TGRA for gist like memory consolidated in the neocortex. Nadel and Moscovitch (1997) in this instance posits that a flat gradient of episodic memory results when the memory did not get enough time to consolidate in non-HPC structures. Moreover, TGRA results with complete HPC lesion because a gist-like representation of the memory gets consolidated in the neocortex. Tenets of the MTT, however, have been falsified (Sutherland et al., 2008; Lehmann, Clark, & Whishaw, 2007). There are studies that suggest that partial lesions can impair remote more than recent memories. Additionally, complete damage has failed to disrupt memories that are believed to be episodic.

This will be discussed in section 1.5. The SMSC and MTT, have been longstanding and influential. However, a growing body of evidence fails to support either of these systems consolidation views. Here are some of the key studies that cannot be accounted for by current systems consolidation theories.

1.4 Disconfirming the SMSC

There are human and non-human studies that report equivalent forgetting for recent and remote memories (flat gradient as opposed to TGRA). Just to recap, the SMSC posits that a memory will eventually consolidate to neocortical structures and thus will be spared after HPC damage. So, studies that find flat gradients after HPC damage, over an extended period of time, should discredit this theory. In fact, Sutherland et al. (2008) reported a flat gradient using CFC in rats. Rats received a foot-shock that was immediately preceded by a tone; they then received either sham or HPC lesions at a 2 days or 12 weeks after conditioning. They reported that damage to the HPC caused RA for learned fear of a context regardless of how long before surgery the learning episode took place (Sutherland et al., 2008). This study provides evidence against the standard model of system consolidation because it did not show TGRA for remote memories. Bolhuis et al. (1994) reported the same flat gradient using a spatial navigation task. Rats were placed in a circular swimming pool starting at one of five positions along the side of the tank. Each rat was given 60 seconds to find a submerged platform. If the rat did not find the platform during that time, it was guided to it by hand. Following initial training, they were given HPC or sham lesion either 14 weeks or 3 days after. They reported equivalent forgetting for the recent as well as the remote time point. Bolhuis et al. (1994) explained that their result does not support the existence of a time dependent consolidation process (Bolhuis, Stewart, & Forrest, 1994).

A closer look at Patient H.M.'s amnesia, who had HPC damage, reveals that he actually did not have TGRA, but instead showed evidence of a flat gradient. Corkin (2005) explains that at H.M's high school reunion (after the removal of his medial temporal lobe including his HPC), a number of his classmates remembered him and greeted him warmly, but he did not recognize anyone's face or name. Milner et al. (1968) had reported that H.M.'s retrograde amnesia (RA) was restricted 2 years preceding his operation. But Corkin emphasized that this conclusion was based on information from the neurosurgeon's office and his mother which was inaccurate (Corkin, 2005). Here again, a flat gradient was reported and a time limited role of the HPC in consolidation was not evident.

The SMSC might argue that the memories that were lost following HPC damage did not get enough time to consolidate to neocortical structures. But, notice that in the cases reported above, some report TGRA 2 years before HPC damage, while others suggests 14 to 12 weeks. The same variation in the learning-to-surgery interval is also reported in other studies. For instance, some studies show TGRA for remote memories acquired 1 day before HPC damage while others report TGRA for memories acquired as long as 199 days (Quinn, Ma, Tinsley, Koch, & Fanselow, 2008; Tse et al., 2007). Hence, the argument that flat gradients result because the memories did not get sufficient time to "transfer" to neocortical structures is not a good one.

1.5 Disconfirming the MTT

The MTT that was proposed to explain the shortcomings of the SMSC, proved to have some shortcomings of its own. As previously mentioned, one way to challenge the MTT theory is to show partial HPC damage does not result in TGRA (Lehmann et al., 2007; Sutherland et al., 2008 & Maren et al., 1997).

Lehmann et al. (2007) showed that partial HPC damage produces a reverse TGRA, meaning memories are lost for remote but not recent memory (Lehmann, Lacanilao, & Sutherland, 2007). After rats were trained in a contextual fear conditioning task, they received sham or partial HPC surgery 1 week, 3 months or 6 months after training. This study directly discredits the MTT. According to the MTT these rats should have showed TGRA thereby showing spared remote memory. The MTT predicts that partial HPC damage will affect recent memories more than remote memories, but this was not what Lehmann et al. (2007) found. Similarly, Sutherland et al. (2008) found a reverse gradient following dorsal or ventral HPC damage. They trained rats using a context paired a foot shock, 2 or 12 weeks later, rats received either sham, dorsal or ventral HPC lesions. They reported that HPC lesioned rats showed more severe RA for remote than for recent memory.

Clearly, there are some discrepancies in the literature for TGRA. There is some evidence of TGRA but there is also evidence of equal forgetting of recent and remote memories (flat gradients) (Lehmann et al., 2007; Sutherland et al., 2008; Bolhuis et al., 1994). This brings us to a crossroad, both findings might be true but the explanations given by the MTT and SMSC are not strong and do not withstand scrutiny. Instances in which there is an observable gradient, might not be because of time at all, but rather another factor—reinstatements. The next section explains the parameters surrounding this suggestion.

1.6 To resolve the discrepant findings across studies showing TGRA and reverse TGRA

In response, at least in part, to the failures to find evidence for TGRA following HPC damage, Sutherland et al. (2010) examined an alternative possibility that may contribute to memories becoming resistant to HPC damage: reinstatements. They explained that a learning

encounter creates a strong representation of the memory in the HPC and a weak representation in non-HPC structures. If learning sessions are distributed or reinstated over several sessions, each reinstatement will incrementally enhance non HPC representation of relevant information, so much that the non-HPC structures would be able to support such memories in the absence of the HPC (Sutherland et al., 2010). Several other theorists also argued that exposure to multiple similar experiences tends to overcome the necessity of the HPC for retrieval of memories (McClelland, McNaughton, & O'Reilly, 1995; McDonald et al., 2002; O'Reilly & Rudy, 2001; Sherry & Schacter, 1987). They emphasized that several repetitions of a learning episode may be required for neural changes in non-HPC networks to occur to reach sufficient strength to result in observable learning behavior, without an intact HPC. There is compelling evidence supporting the Distributed Reinstatement Theory (DRT), some of which will be described in the next section.

1.7 Support for the DRT

Sutherland et al. (2010) provided a theoretical account of the DRT but there is also empirical evidence supporting this view (Lehmann et al., 2009). Lehmann et al. (2009) placed rats in a conditioning chamber where they received a shock (shock context). Forty-five minutes later, the same rats were placed in a different chamber for the same amount of time without receiving any shocks (no-shock context). This was repeated two times a day for five consecutive days. Rats were then given HPC or sham surgery. When the rats' retention was assessed in both contexts sham rats in the no-shock context regardless if they had HPC lesion or sham surgery displayed a memory deficit. Importantly, rats in the shock context regardless of surgery type (HPC or sham) displayed memory of the context. Another group of rats received the same number of shocks, spent the same amount of time in the conditioning chamber as the previous rats, the time

between the conditioning session and surgery matched—the only difference in this group of rats is that they were given a single conditioning session, while the previous group received distributed leaning sessions. Sham rats in the single learning session showed fear of the context and therefore intact memory, but rats with HPC lesions had retrograde amnesia. Lehmann et al. (2009) went on to explain that these findings suggests that a context fear memory that is normally dependent on the HPC are incrementally strengthen in other non-HPC structures with distributed learning, a similar theoretical explanation given by Sutherland et al. (2010) (Lehmann et al., 2009). It showed no evidence of consolidation but distributing the learning made the memory HPC independent. This evidence does not provide much support for the idea that there is a lengthy process of systems consolidation following a learning episode. Instead, distributing the learning episodes allows the memory to quite quickly survive HPC damage. Importantly, there are replications showing the mitigating effects of reinstatements on retrograde amnesia following HPC lesions (MacLeod, Reynolds, & Lehmann, 2018; Lehmann & McNamara, 2011).

There is also a case study that supports the DRT. Patient T.T. was a London Taxi driver who had bilateral HPC damage following limbic encephalitis (Maguire et al., 2006). He worked as a London taxi driver for nearly 40 years. London taxi drivers go through extensive training, learning the layout of 25000 streets in the city. Using a video game of a virtual reality of London, T.T. was impaired on some routes but not others. Those routes that remained intact were the ones he navigated more frequently and was hence repeated more often (Maguire et al., 2006). This case provides support for the DRT because it can be proposed that those routes that remained intact were the ones that were reinstated many times. Those routes that are lost were not reinstated enough to create a sufficiently strong enough representation in non-HPC structures to survive HPC damage.

Also, going back to my initial story with Mr. Bob who remembered the shit epidemiology so vividly to tell it passionately and make me believe it, it is possible that he remembers it not because of how old the memory is, but because he had told it so many times. It is possible that this memory was reinstated many times and made less vulnerable to HPC damage.

1.8 Issue with the DRT

The DRT posits a plausible, supported model, but there is an aspect of the DRT that has been overlooked—the role of the saliency of the learning episode. It does not address the role of saliency of the learning episode in making an HPC-dependent context fear memory into an HPC-independent one. Independently, highly salient memories create robust memories (Fanselow, 1980; McAllister, Dieter, & James, 1979) and distribution of a memory can enable a memory to become HPC-independent (Lehmann et al., 2009; Lehmann & McNamara, 2011). But, there is no thorough investigation about whether a context fear memory needs to be highly salient and be acquired via distributed learning session to mitigate retrograde amnesic effects, or whether any memory, independent of saliency or distribution, will mitigate retrograde amnesic effects. This is an important aspect that should not be overlooked and what was addressed in this thesis.

1.9 Aim of this Thesis

This thesis examines whether a combination of distribution of the learning episodes and high saliency is necessary to mitigate retrograde amnesic effects and create a HPC independent memory. The experiments in Chapter 2 aimed to determine whether any contextual fear memory distributed over several sessions will become HPC independent or whether the saliency of the memory plays a role too. If distribution of the memory alone is necessary to make the context fear memory survive HPC damage then HPC lesioned rats that receive distributed learning

should display fear (high freezing) of the high, medium *and* low salient conditions. If saliency is required in addition to reinstatements, then the high saliency condition should mitigate the retrograde amnesia the most. In chapter three, the neocortical structures that come to support highly salient distributed were examined. Indeed, candidate regions that might support the reported HPC-independent for context fear memory in Chapter 2 were examined for IEG expression (c-fos). Finally, Chapter 4 discusses the overall implications and theoretical impact stemming from the findings of this thesis.

Chapter 2: The role of saliency in making a memory HPC independent

2.1 Introduction

Sutherland et al. (2010) proposed that a HPC-dependent memory can become HPC-independent over several reinstatements and this has been compellingly demonstrated for a context fear memory in rats (Lehmann et al., 2009). Indeed, damaging the HPC after distributed context-fear conditioning no longer caused retrograde amnesia. Lehmann et al. (2009) gave rats 11 learning episodes distributed over 6 days. In each session, the rats were placed in a context and received a foot-shock (shock context). Forty-five minutes after they were placed in another context but did not receive any shock (no-shock context). The procedure was repeated twice daily for 6 consecutive days and the shock and no shock chamber were counterbalanced. One group of rats then received sham surgery while another group received HPC damage 1-3 days later. The rats' retention was assessed in both contexts. HPC-lesioned rats in the no-shock context did not freeze, as expected however, HPC lesioned rats in the shock-context showed comparable freezing to sham rats, suggesting a memory of the context. Moreover, another group of rats was trained in a single learning session in which they received the same number of shocks, context exposure time and interval between initial learning and surgery as the previous group. HPC lesioned rats in this group showed RA when compared to the sham rats. Together these findings suggested that distributing the learning episodes allowed the context fear memory to become increasingly reliant on non-HPC memory structures and can therefore be expressed without it. It is unclear, however, whether distribution of the conditioning episodes alone is sufficient or whether a combination of distribution and high saliency shock is necessary to make the context fear memory become HPC independent. In fact, there is evidence that saliency is important for making memories stronger and more likely to be remembered following brain

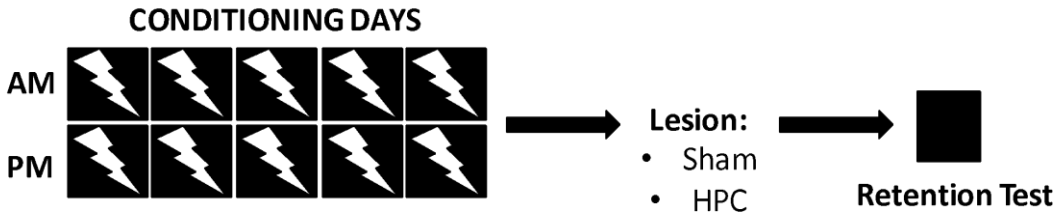
disruption (Fanselow, 1980; McAllister, Dieter, & James, 1979; Quiroz et al., 2003; Cobos-Zapiain et al., 1996; Giordano & Prado-Alcala, 1986).

In the current chapter, the experiments aimed at determining whether distributed reinstatements alone are sufficient to enable the formation of an HPC-independent memory or whether saliency of the learning episodes (shock intensity) is also a critical factor. In the first experiment, context fear learning occurred over distributed conditioning sessions, which is known to establish an HPC-independent memory. However, the distributed conditioning involved one of three different learning saliencies: low, medium, or high foot-shock intensities. If distribution of the memory alone is sufficient to make the context fear memory become HPC independent, then complete HPC lesions should fail to cause retrograde amnesia for the context fear memory regardless of the high, medium, or low conditioning saliencies. If, however, saliency is required in addition to reinstatements, then the high saliency condition should mitigate the retrograde amnesia the most.

Another experiment was also conducted to confirm that reinstatements are necessary beyond high saliency conditioning. In this instance, high saliency conditioning occurred in a single session (Massed Conditioning) rather than across distributed reinstatements. Importantly, the context fear conditioning parameters, such as the number of context shock pairings, the amount time in the context, the conditioning-to-surgery interval were all matched to the previous experiment. Hence, the difference between this experiment and Experiment 1 resided in learning in a single massed session vs. across distributed reinstatement sessions. Given that several studies have demonstrated that contextual fear conditioning acquired in a single session and fails to make a memory HPC-independent, it was expected that complete HPC damage would cause retrograde amnesia and thus confirm that reinstatements are critical for making a memory

become HPC-independent. The experimental design for both experiments is illustrated in Figure 2.1.

A) Distributed Conditioning



B) Massed Conditioning

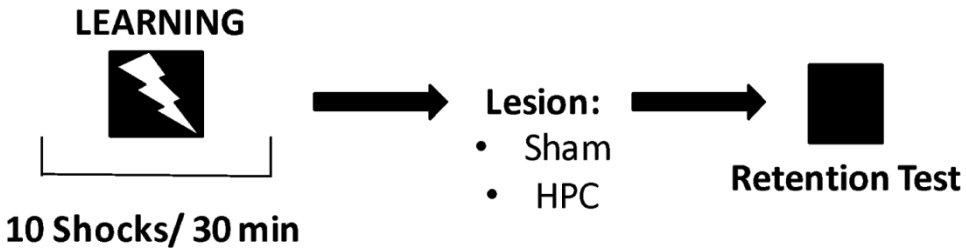


Figure 2.1. In the Distributed Conditioning experiment (A), the rats received 10 contextual fear conditioning sessions (3 mins) across 5 consecutive days. In each session, the rats received a single shock, but the rats were assigned to receive either low (0.4 mA), intermediate (0.7 mA) or high (1.0 mA) saliency/intensity shocks. Then, the rats from each shock-saliency group received sham or HPC lesions. Approximately 10-14 days after the surgery, the rats were given a retention test. In the Massed Conditioning experiment (B), the rats were given a single conditioning session (30 mins) in which they received 10 high saliency shocks (1.0 mA). They were then given either sham or bilateral HPC lesions. Approximately 10-14 days after surgery, the rats were given a retention test.

2.2 Methods

2.2.1 Subjects

All procedures were approved by the Trent University Animal Care Committee, which follows the guidelines set by the Canadian Council on Animal Care. The subjects were 55 male Long Evans rats (Charles River, Quebec) and 3-4 months old at the beginning of behavioural training. Three rats were excluded because of acquisition issues, leaving a total of 52 rats in the study. The rats were housed in groups of two in ventilated-laboratory cages and maintained on a 12:12-hr light-dark cycle (lights on at 0700 h). Each rat received 25-30 g of rat chow daily and had access to water *ad libitum.*

2.2.2 Apparatus

A Ugo Basile (Varese, Italy) conditioning chamber was used across all experiments. The chamber measured 25.4 x 25.4 x 36.5 cm and was made of Plexiglas, with a circular front opening door. The floor consisted of 21 metal rods (3 mm diameter), spaced 1.2 cm apart center to center. The conditioning chamber was also housed in a sound-attenuating chamber (54.3 x 46.4 x 55.1 cm). The shocks were delivered through the metal rods in the floor, which were connected to a shock generator and scrambler (Ugo Basile, Varese, Italy). The chamber was 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, Wood Dale, IL) was used to conduct the context fear testing and behavioral scoring. 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). For this, the program parameters were set at a sensitivity of 70 for 33 freezing onset and freezing 80 for offset. In addition, a rat was considered freezing only after 250 ms 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 memory.

2.2.3 Procedure

2.2.3.1 Conditioning

Distributed: The rats were individually transported in a plastic bucket (24.0 x 24.3 x 33.6 cm) to contextual fear conditioning room and placed inside the conditioning chamber. Each rat received 10 conditioning sessions distributed across 5 consecutive days. On each day the rats received a 3-min conditioning session in the morning (9-11 AM) and another 3-min session in the afternoon (12-2 PM) (see figure 2.1A). During each session, the rats received either a 2 sec 0.4 mA (low saliency), 0.7 mA (medium saliency) or 1.0 mA (high saliency) shock at the 2 min mark. Immediately after each session, the rats were returned to their home cage.

Massed: The rats that received the high salient shock in a single conditioning session were transported to the conditioning room the same way previously described. They received 10 context-shock pairings (1.0 mA/2 sec), however, within a single 30 min session (see figure 2.1 B). Initial shock onset was set at 120 sec, with a recurring shock every 3 min thereafter.

2.2.3.2 Surgery

Seven to nine days after the first conditioning session, the rats received either Sham or HPC damage. It is important to note that the interval between the first conditioning session for the distributed and the single session for the massed condition was matched. This was done to avoid arguments of systems consolidation (Kim & Fanselow, 1992; Nadel & Moscovitch, 1997). The rats' assignment to either the sham or HPC surgery group in the distributed condition was matched on their pre-shock freezing behaviour on the last day of conditioning. Similarly, rats in the massed condition was assigned to either the sham of HPC surgery group based on their total percentage of time spent freezing across the conditioning session. This was followed to ensure that any potential post-operative/retention test differences could not be accounted for by differences in pre-operative/conditioning freezing levels.

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) as well as an anticonvulsant (gabapentin, 0.4 ml; 100 mg/ml, i.p.; Chiron, Guelph, Ontario). The rats were then placed in a stereotaxic frame (Stoelting, Wood Dale, IL). An incision was made along the midline of the scalp, which was then retracted to expose the skull and bregma. For the HPC lesions, small burr holes were drilled at six sites bilaterally (see Table 2.1) and an *N*-methyl-D-aspartic acid (NMDA; 7.5 µg/µl, in 0.9% physiological saline, Sigma Chemical, St. Louis, MO) was injected across the HPC to damage the structure. The injections were performed using a 30 Ga needle attached to a 10 µl Hamilton syringe via polyethene tubing (PE-50). The NMDA was injected into the HPC at a rate of 0.4 µl/min and the volume varied between 0.3-0.4 µl depending on the injection site (see Table 2.1)

and controlled by a microinfusion pump (KD Scientific, Holliston, MA). The injection needle remained in place for 2 min after each injection to allow diffusion of the NMDA. After the completion of all injections, the incision was sutured. For 7 d following surgery, the rats were given an oral analgesic (Metacam, Oral Suspension 0.1 ml; 1.5 mg/ml, p.o.; Boehringer-Ingelheim) daily.

Table 2.1. Stereotaxic injection coordinates relative to Bregma (mm) and volume of NMDA infused at each site.

Anteriorposterior (AP)	Mediolateral (ML)	Dorsoventral (DV)	Infusion Volume (µL)
-3.0	±1.5	-3.6	0.3
-4.0	±3.0	-4.0	0.3
-4.9	±3.0	-4.0	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	0.4

2.2.3.3 Retention

Ten to 14 days after surgery, the rats were returned to the conditioning context for a 5-min retention test. Transportation procedure was the same as those described in the Conditioning section. Note that no shock was delivered during this test.

2.2.3.4 Histology

Sixty minutes following the completion of behavioural 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 then sectioned at a thickness of 40 μm 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. Digital images of each section were then taken at a 2X magnification using a light microscope (Nikon H600L), camera (DS-Qi1Mc), and Nikon Element software (Nikon Instruments Inc., Melville, NY), in order to enable quantification of the lesions.

The HPC lesion extent in each rat was estimated according to the Cavalieri and point-counting principles (Schmitz & Hof, 2005). Using ImageJ software
37 (http://rsb.info.nih.gov/ij/), a sampling grid with an area per point of 0.05 mm2 was randomly superimposed on each digitized section. HPC images that were quantified were taken at a 2X magnification using a light Nikon H600L microscope with a DS-QilMc camera and Nikon Element software (Nikon Instruments Inc., Melville, NY). The HPC was defined as spanning from -1.72 mm to -6.72 mm relative to Bregma (Paxinos & Watson, 2006). Grid points that intersected the HPC cell fields (CA1-3, hilus, faciolarumcinereum and dentate gyrus; 10-12 sections per brain) were counted for each section. The total number of points counted in the HPC

for each brain was then divided by the average count from 4 control rats (Mean = 443.0, SD = 43.4) and multiplied by 100 to produce an estimate of the percent of remaining tissue, the complement of which corresponded to the lesion size.

2.3 Results

2.3.1 Histology

The neurotoxic injections of NMDA produced extensive damage to the HPC in 30 of the 34 rats that received the lesions. The data of these four rats, all from the massed condition, were excluded because they had less than 50% HPC damage, which is demonstrated to be insufficient to cause retrograde amnesia (Scott, Saucier, & Lehmann, 2016). Thus, 9 rats remained in this Massed-HPC group. Table 2.2 provides descriptive statistics of the lesion estimates for each lesion group, whereas Figure 2.3 illustrates an average lesion of the HPC. Importantly, an one - way ANOVA indicated that the lesion sizes did not statistically differ across groups $F(3, 27) = 0.56$, ($p = .29$). For the HPC group, the NMDA injections produced extensive neural loss to all HPC cell fields bilaterally. One rat in the high saliency condition had posterior bilateral sparing of the CA1 and CA2 fields. There were four rats with unilateral sparing of the dentate granule cells. Three rats in the massed condition had minimal posterior bilateral sparing of the CA1 and CA2 pyramidal cells. All rats had minor damage to the posterior parietal cortex where the injection cannulae were inserted and some rats sustained minor damage to the fimbria/fornix. No damage was found in the thalamus, amygdala or rhinal cortex.

Table 2.2. Descriptive statistics for the percentage of HPC damage in lesion rats included in the distributed and massed conditions.

	HPC Damage (%)				
Group	Mean	St. Dev.	Smallest	Largest	n
Massed					
1.0 mA	78.9	5.5	71.1	90.1	9
Distributed					
0.4 mA	86.4	4.5	80.1	90.9	6
0.7 mA	86.9	5.3	81.2	95.5	7
1.0 mA	89.1	4.0	84.2	95.2	8

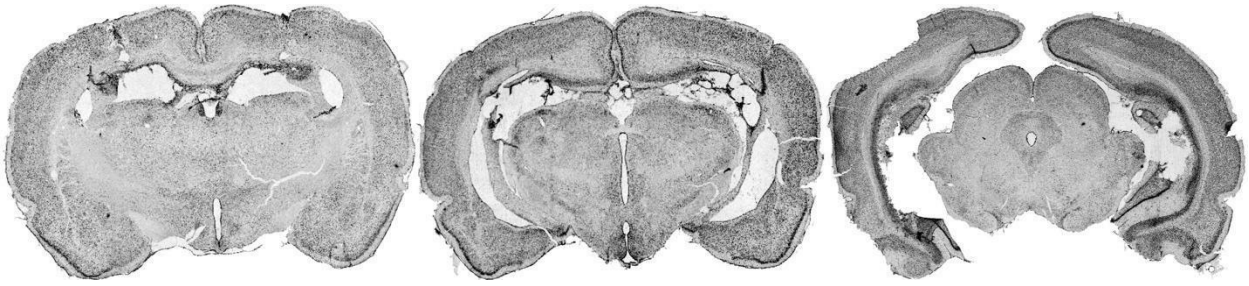


Figure 2.3. Photomicrographs (2X) of coronal brain sections showing an average (88%) lesion of the HPC. The damage was complete in the dorsal region (first two images) and very little was spared in the posterior/ventral region (third image).

2.3.2 Behavioural Results

2.3.2.1 Distributed

2.3.2.1.1 Conditioning. Figure 2.4 depicts the pre-shock freezing across conditioning sessions for each shock intensity. A mixed design ANOVA with conditioning sessions (1-10) as a within-subject factor and saliency (0.4, 0.7., and 1.0 mA) as a between subject factor revealed a significant interaction, ($F(18, 306) = 6.169$, $p > .05$). Thus, the pattern of freezing across sessions differed across saliencies. To breakdown the interaction, comparisons on freezing between the first and last conditioning sessions for each saliency were conducted to determine whether context freezing was higher at the end versus the beginning of conditioning. The pairwise comparisons revealed that the rats in the 0.7 and 1.0 mA condition showed significantly more freezing at the end ($p < 0.05$), whereas the rats in the 0.4 mA condition did not ($p = 0.155$). Thus, the rats in the intermediate and high saliency condition showed context fear conditioning, but not the rats in the low saliency condition. The main effect of saliency was also significant ($F(2, 34) = 37.431$, $p > .05$), showing that the higher the intensity increased freezing and this was supported by the pairwise comparisons between each intensity ($ps < 0.05$). The main effect of session was significant ($F(9, 306) = 35.296$, $p > .05$), suggesting that freezing increased over sessions. This effect, however, was not analyzed any further because our interaction analysis sufficiently addressed the pattern over sessions.

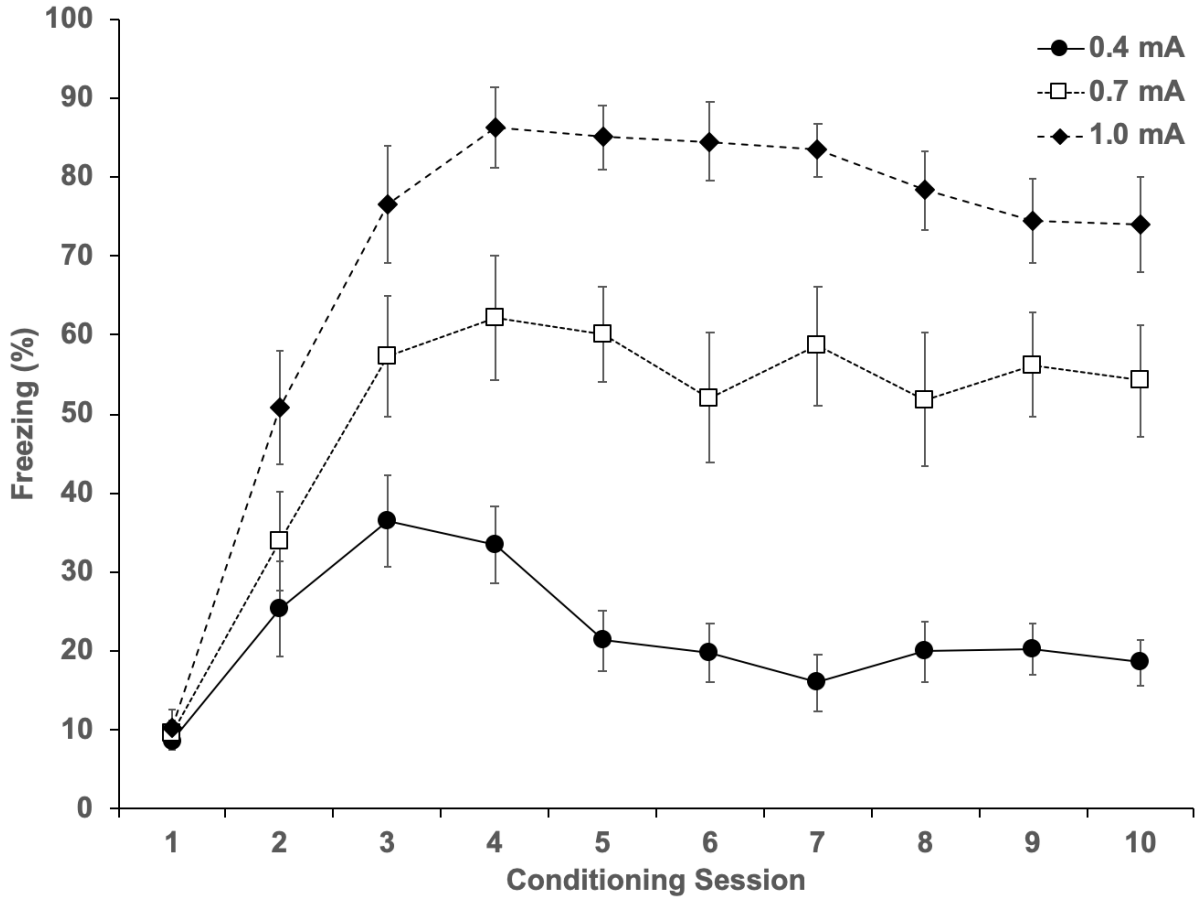


Figure 2.4. Mean (±SEM) pre-shock freezing percent across conditioning sessions for each shock intensity group. Overall, the rats in the Intermediate (0.7 mA) and High (1.0 mA) saliency conditions associated the context with the shock because their freezing was significantly higher in the last session compared to the first (ps < 0.05). The rats in the Low saliency condition, however, showed minimal freezing across sessions suggesting poor, if any, context fear conditioning. Also, the conditioning was the strongest in the High saliency group ($p < 0.05$).

Importantly, the percent time freezing for HPC and Sham rats in the low (0.4 mA), intermediate (0.7 mA), and high (1.0 mA) saliency conditions were matched prior to surgery according to their pre-shock performance on the last day of conditioning (Day-5 AM and PM sessions combined). These data are illustrated in Figure 2.5. A 2 X 3 between-subjects ANOVA, with Surgery (Sham, HPC) and Saliency (0.4, 0.7, and 1.0 mA) as factors only revealed a significantly main effect of Saliency, $F(2, 31) = 24.715$, $p < 0.05$. The interaction was not

significantly meaningful, F (2, 31) = 0.91, p = .914, nor was the main effect of Lesion, F (1, 31) = 0.007, p = .936. Thus, in each saliency condition, the lesion groups were properly matched and the strength of conditioning increased with increasing saliency.

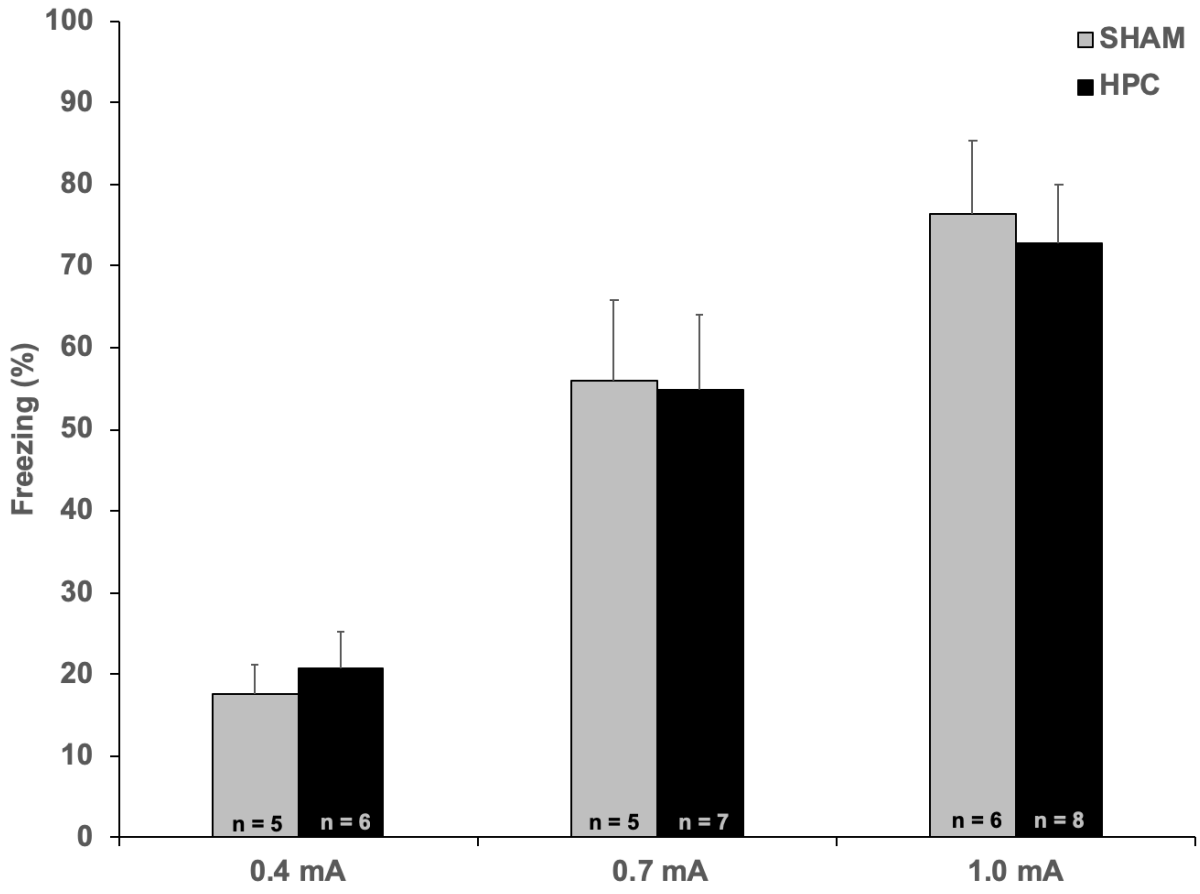


Figure 2.5. Mean (+SEM) percent time freezing on the last day of conditioning (Day-5 AM and PM sessions combined) for HPC and Sham rats in the low (0.4 mA), intermediate (0.7 mA), and high (1.0 mA) saliency conditions. There was no statistically significant difference between rats assigned to receive HPC or Sham lesions in either condition (ps > 0.05), suggesting that they were properly matched for acquisition prior to their surgery.

2.3.2.1.2 Retention. Figure 2.6 illustrates the freezing data from the retention test. A 2 X 3 between-subjects ANOVA, with Surgery (Sham, HPC) and Saliency (0.4, 0.7, and 1.0 mA) as factors, failed to reveal a significant interaction, $F(2, 31) = 1.118$, $p = .34$. Nevertheless, because of our design and predictions, the data were analyzed with planned pairwise comparisons. Least Significant Differences (LSD) tests indicated that the HPC rats from the 0.7 mA group showed statistically less freezing than their respective Sham group ($p < 0.05$). This finding suggests that the HPC lesions caused retrograde amnesia and that distributed fear conditioning involving shocks of intermediate saliency remained dependent on the HPC. In contrast, the HPC rats from the high saliency condition showed high levels of freezing and did not statistically differ from their respective sham group ($p = 0.492$), suggesting that the distributed conditioning with a high intensity shock became HPC-independent. No meaningful statistical difference was found between the HPC and sham rats in the low saliency condition (0.774). However, this comparison is not very meaningful given the conditioning was extremely poor for this shock saliency (see pre-surgery conditioning). Moreover, the sham rats in this condition did not show significantly more, though approaching significance, freezing to the context during the retention test then they did prior to ever receiving a shock during conditioning (pre-shock session 1), $t(4) = 2.702$, $p = 0.054$. Although this difference is close to being significant, the amount of freezing remains extremely low and context fear memory cannot be inferred from the freezing data in the low saliency condition.

The pairwise analyses provided other useful information beyond comparisons between the HPC and Sham groups. First, the freezing significantly increased in the sham rats with increasing shock intensities. Indeed, the High froze more than the two other groups, and the Intermediate more than the Low ($ps < 0.05$). Similarly, when comparing the HPC groups, the

HPC-High showed significantly more freezing than the HPC-Low and HPC-Intermediate groups (ps < 0.05), whereas no statistical difference was found between the HPC-Intermediate and HPC-Low (p = 0.275).

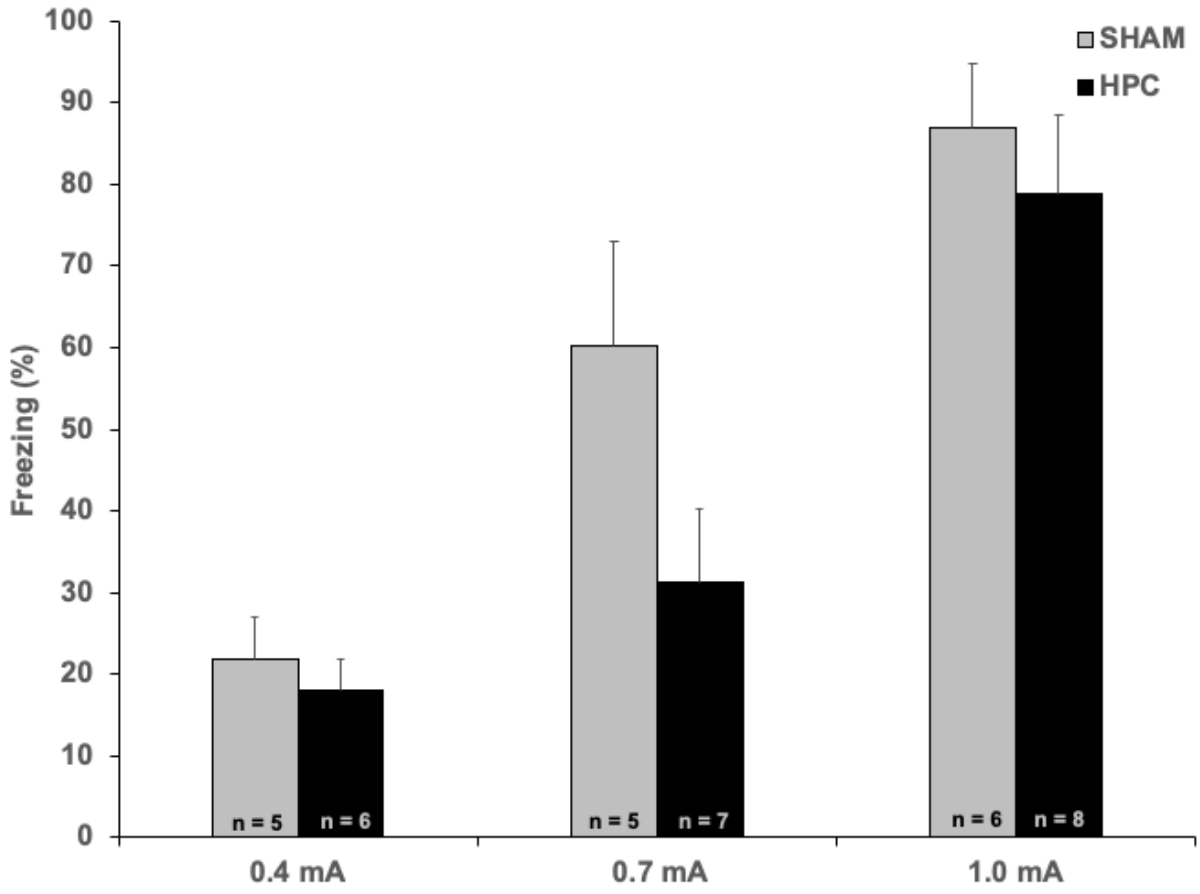


Figure 2.6. Mean (+SEM) percent time freezing on the retention by Sham and HPC from each distributed conditioning saliency condition. The rats, Sham and HPC, from the Low saliency (0.4 mA) showed poor freezing and no meaningful context fear memory. The HPC rats from the Intermediate saliency condition (0.7 mA) showed significantly less (p < 0.05) freezing than their respective Sham control group, suggesting that they suffered from retrograde amnesia. In contrast, the HPC rats from the High saliency condition (1.0 mA) showed comparable freezing to their respective control group and thus did not suffer from retrograde amnesia. These findings suggest that a high saliency shock is required to make distributed context fear memory become independent of the HPC.

The main effect of Saliency was significant, $F(2, 31) = 26.566$, $p < 0.05$), which is result mainly driven by the Sham rats (see above). The main effect of Surgery was not statistically significant, $F(1, 31) = 3.555$, $p = .069$). However, this was likely because of the poor conditioning in the Low saliency condition.

2.3.2.2 Massed

2.3.2.2.1 Conditioning. The rats that received the massed conditioning session showed very low levels of freezing prior to receiving their first shock ($M = 4.62$, $SD = 6.41$) and statistically higher levels after their last shock ($M = 55.02$, $SD = 24.59$; $t(14) = -7.579$, $p < 0.05$). In addition, the rats assigned to the HPC ($M = 58.00$, $SD = 14.91$) and Sham ($M = 66.28$, $SD = 18.71$) groups were matched for overall session freezing and did not statistically differ, $t(13) = 0.954$, $p = 0.358$.

2.3.2.2.2 Retention. Figure 2.7 illustrates the freezing data on the retention test of the rats that received massed conditioning. A t-test revealed that the HPC group froze significantly less than the Sham group, $t(13) = 8.813$, $p < 0.05$. This finding suggests that the post-conditioning HPC lesions caused retrograde amnesia for a context fear memory acquired in a single session.

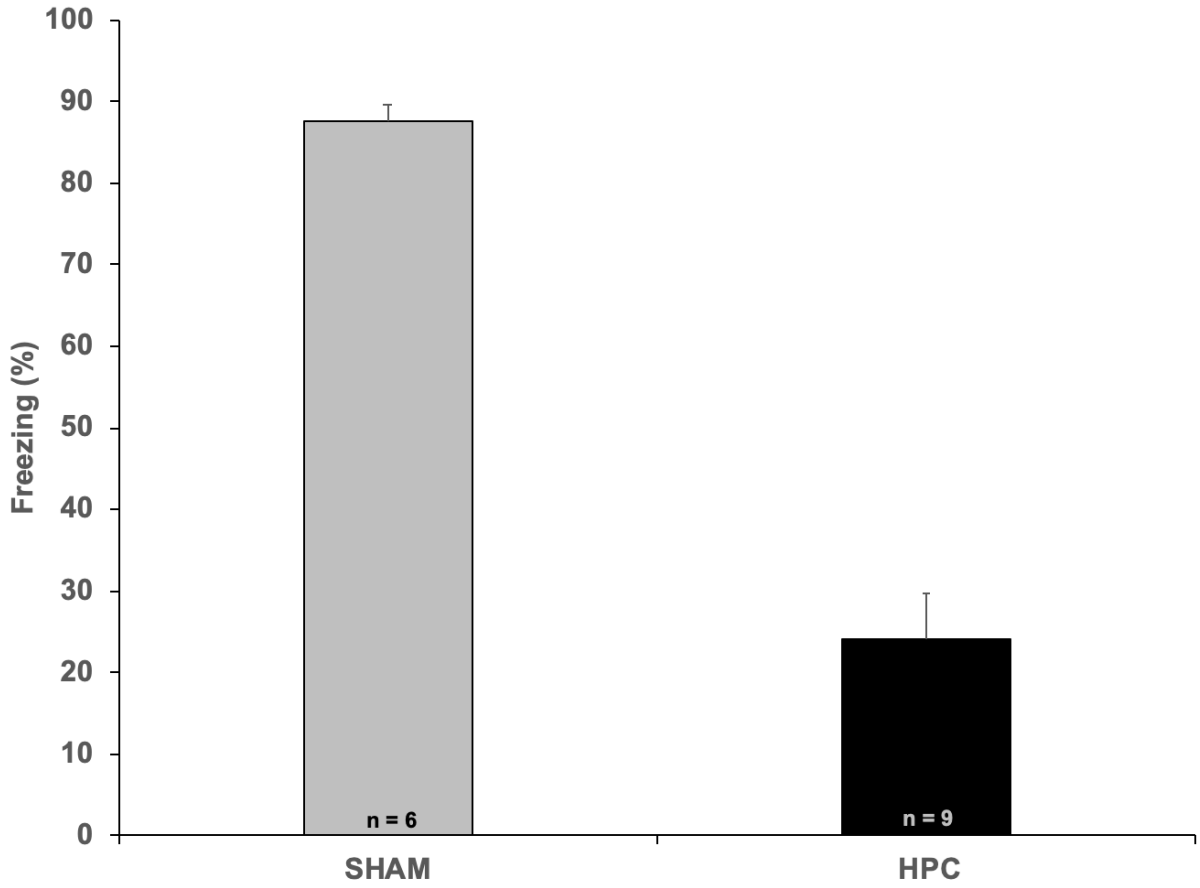


Figure 2.7. Mean (+SEM) percent time freezing on the retention by Sham and HPC from the massed condition. The HPC rats froze significantly less than the Sham rats ($p < 0.05$), suggesting that the lesions caused retrograde amnesia. It is important to note that during conditioning, these rats received 10 context-shock (1.0mA) pairings in a single massed session, which matches the context-shock parameters from the high saliency group from the distributed condition. Yet, unlike in the Distributed condition, the lesions in this instance caused retrograde amnesia, suggesting that distribution of the high saliency shock is necessary to make the context fear memory become independent of the HPC.

2.4 Discussion

This experiment examined whether distributed reinstatements alone is sufficient to enable the formation of an HPC-independent memory or whether saliency of the learning episodes (shock intensity) is also a critical factor. It was demonstrated that distributed conditioning made an HPC-independent memory, but only when it involved conditioning with a high saliency/high

intensity shock, thereby suggesting that the saliency of the learning episodes is indeed a critical factor in forming an HPC-independent memory. The spared memory for a context fear memory after complete HPC lesions replicate and confirm those of other studies demonstrating that distributed reinstatements can make a memory become HPC-independent (Lehmann et al., 2009; Lehmann & McNamara, 2011). However, distribution of the conditioning is insufficient. Indeed, in the intermediate saliency condition, the sham rats showed good retention of the context fear memory, but the HPC-lesioned rats did not. Unfortunately, the low saliency condition did not offer strong enough conditioning to even infer memory in the control rats. The levels of freezing at the end of conditioning was no higher than prior to even receiving their first context-shock pairing (naïve state) and remained at similar low level on the retention test. Hence, the null effect of the lesions in this condition is meaningless. However, the data from both groups in the low saliency condition can be used, to some extent, as a baseline measure of freezing for rats with no context fear memory. Importantly, when using the HPC-Low rats as a non-conditioned group, then the amnesia in the intermediate group can be interpreted as a complete loss of the memory because their performance was no better. The intermediate saliency shock was strong enough to create good conditioning however, not strong enough to establish an HPC-independent memory. HPC lesion rats as well as Shams in the high saliency distributed condition displayed high levels of freezing during retention, thereby suggesting intact context-fear memory. Note that the rats in this condition received the same intensity foot-shock (1.0 mA) as those in the massed condition. Nevertheless, the HPC lesion rats in the massed condition had retrograde amnesia while those in the distributed condition did not. This suggests that a highly salient context fear memory alone is insufficient to create a HPC-independent memory and that the context-shock pairings require a sufficiently intense shock as well as distribution to mitigate retrograde amnesic effects of HPC

damage. Surprisingly, the group that showed spared memory following the lesions (distributed 1.0 mA) was the one with the largest amount of damage. The rats that underwent massed conditioning had an average lesion size 78.9% which is less than those rats that experienced the distributed conditioning session (table 2.1). Hence, even with extensive HPC damage, a memory that is highly salient and distributed becomes HPC-independent.

Sutherland et al. (2010) proposes that the HPC interferes with or overshadows memory acquisition by other systems. There are a number of studies that demonstrate that there is retrograde amnesia following HPC damage but memories are unaffected in the anterograde direction (Ross & Eichenbaum, 2006; Sara, 1981; Maren et al., 1997), meaning post-learning HPC lesions results in retrograde amnesia but animals have no problem acquiring the memory when the HPC is damaged. Sutherland et al. (2010) proposes that the fact that multiple, distributed learning episodes can overcome this overshadowing is consistent with a parallel dual-store theory where each learning episode triggers a short period of memory replay that provides a brief HPC dependent systems consolidation. With each learning session, the memory is incrementally strengthened in the neo-cortex thereby supporting the expression of it. In light of the new information gathered from this experiment, it is reasonable to suggest that stronger context fear memories somehow produce larger, more lasting incremental representation of the memory in the neocortex, thereby producing a memory that is not vulnerable to HPC lesion. But, what structures now support these context fear memories? In the next chapter, we examine the non HPC structures supporting the expression of these highly salient fear memories, by using c-fos, an IEG, that is upregulated with increased neuronal activity.

Chapter 3: Identifying non-HPC structures that support highly salient context fear memories

3.1 Introduction

The DRT suggests that repetition of an event or repeated memory reactivations can strengthen a memory in non-HPC systems to the point that it no longer requires the HPC for recall (Lehmann et al., 2009; Sutherland et al., 2010). Hence, a memory can transition from an HPC-dependent to independent state over reinstatements. In the previous chapter, it was found that contextual fear acquired over distributed sessions and with a salient reinforcer (shock) enabled a context fear memory to become independent of the HPC and withstand complete HPC damage. The context fear memory must then be represented in a non-HPC network/system, but what is this system? In other words, what structures are supporting the context fear memory in absence of the HPC?

In the current chapter, the experiment aimed to determine whether three structures, the perirhinal cortex (PRH), the basolateral-amygdala (BLA), and the anterior cingulate cortex (ACC), contribute to supporting the HPC independent memory. Each of these structures are known to play a role in context fear memory (Bucci, Phillips, & Burwell, 2000; Einarsson, Pors, & Nader, 2014; Vetere et al., 2011). Indeed, evidence suggests that inactivation or damage to either of these structures causes retrograde amnesia for contextual fear conditioning (Bucci et al., 2000; Corodimas & LeDoux, 1995; Einarsson & Nader, 2012; Ledoux, Cicchetti, Xagoraris, & Romanski, 1990). Also, neural activation studies have shown that these regions show increased activation during context fear recall (Hall et al., 2001; Vetere et al., 2011).

The role of the PRH, BLA, and ACC in supporting context fear memory is, however, believed to be heterogenous. Specifically, each structure is argued to contribute to specific aspect of a context fear memory. For example, the ACC is involved in the processing of pain and unpleasantness of memories. Specifically, the ACC is involved in nociception (Einarsson & Nader, 2012; Tang et al., 2005). There is also evidence that suggests that the ACC is involved in the processing of anxiety and fear (Etkin, Tobias & Raffael, 2011). The PRH is involved in discriminative familiarity of individual stimuli such as recognizing contexts in contextual fear conditioning. Surgical removal of the PRH in monkeys and rats impairs recognition memory for individual objects (Brown & Aggleton, 2001; Meunier, Bachevalier, Mishkin, & Murray, 1993), but more importantly, damage to the PRH before or shortly after training on a contextual fear conditioning task causes deficits in the expression of context fear (Burwell, Bucci, Sanborn & Jutras, 2004). The PRH is reportedly involved in the storage, maintenance and/or retrieval of context fear memory (Burwell et al., 2004). The BLA sub-region of the amygdala was chosen as a region of interest because it is the most systematically explored structure using Pavlovian fear conditioning (Fanselow & LeDoux, 1999), such as the contextual fear conditioning protocol used here. Lesions, whether made before or shortly after conditioning, produce profound deficits in fear to auditory, visual and contextual stimuli (Phillips and Ledoux, 1992; Kim and Davis, 1993). The BLA processes emotional component of memories formed during fear conditioning (LeDoux, 1996; Maren and Fanselow, 1996). Given that the PRH, BLA, ACC play an important role in context fear memory and that they seemingly play different roles, it is hypothesized that they will be increasingly recruited to support a context fear memory that has become HPC independent.

A pragmatic approach to determine whether the PRH, BLA, and ACC support an HPC-independent context fear memory acquired over distributed reinstatements is to examine IEG expression following retention testing of the rats in the previous chapter. As mentioned in the introductory chapter, IEGs are upregulated following neuronal stimulation (Guzowski et al. 1999; Vazdarjanova et al. 2002; Guzowski et al. 2001Vann et al. 2000a; Hall et al. 2001) and can be used to map regions that are involved in a cognitive process such as memory. Moreover, IEG protein signal, like c-fos, up regulation can be detected within 60 to 90 minutes of stimulation, a time at which all the rats were sacrificed after memory testing in the previous chapter. Hence, the brain tissue from the previous experiments was used to assess localization of the HPC-independent context fear memory that was observed in the HPC-lesioned rats that received the high saliency conditioning using the IEG c-fos.

Using the tissue from the experiments in the previous chapter also offered benefits in the experimental design. The structure that is supporting the highly salient distributed memory should show the highest c-fos activation in the HPC lesion rats in the Dist-1.0 mA condition. If the structures are supporting different aspects of the context-fear memory, for instance just the context representation, HPC-lesion rats in the distributed conditions should show elevated levels of c-fos when compared to those in the massed condition or the home-cage controls. Moreover, if the structures are supporting just the fear representation of the memory, there should be high c-fos expression in the HPC lesion rat in the high saliency condition alone.

This experiment assessed the c-fos activation for rats in the distributed high and intermediate salient conditions as well as for the rats in the massed condition. If the BLA, ACC and/or the PRH support highly salient distributed context fear memory then they should show the highest c-fos activation. If there is comparable activation of c-fos in the rats that received the

single conditioning session, the intermediate saliency and high saliency distributed conditioned groups, then these structures do not support the retrieval of the highly salient distributed context fear memory.

3.2 Methods

3.2.1 Subjects

Brain tissue from the rats tested in the experiments of Chapter 2 were used. Additionally, brain tissue from home-cage control rats were included. Doing this gave a baseline c-fos level of experimentally naïve rats.

3.2.2 Procedures

3.2.2.1 Immunohistochemical Procedures

A tissue series ($1/12^{th}$ sectioning sample) from each rat tested was labelled for c-fos and Nissl to quantify neuronal activity in the areas of interest. The tissue from the rats in the low saliency condition, however, was not labeled because the control rats failed to show memory. The tissue from the rats in the intermediate and high saliency conditions as well as those in the massed condition was given an 8-10 min rinse in 0.1% sodium azide, followed by a 24 hr incubation period in a solution of 1:1000 primary rabbit anti-Fos-1 antibody (Santa Cruz Biotechnology) and Triton X. The tissue was then given three 8-10 min rinses in phosphate buffered saline before being transferred to a solution of 1:1000 secondary antibody donkey anti-rabbit Cy3 (red; Jackson Immuno Research Lab) and 1:2000 fluorescent Nissl stain (Neurotrace® green fluorescent Nissl stain; Invitrogen, Eugene, Oregon) for an incubation period of 24 hrs. Sections were then mounted to glass slides and immediately cover slipped using Invitrogen Slow Fade TM Gold (Life Technologies, Burlington, ON).

3.2.2.2 Quantification of c-fos Stereological Procedures – Anterior Cingulate Cortex, Perirhinal Cortex and Basolateral-Amygdala

The estimate for c-fos-positive cells in the BLA, PRH and ACC of each rat was obtained according to unbiased/assumption-free stereology practices using the disector principle (Sterio 1984; Mouton 2002). All stereological quantifications were carried out by an experimenter blind to each animal's treatment history. For each section containing the BLA (6-7 sections per tissue series of a brain), PRH (7-9 sections per tissue series) and ACC (8 to 10 sections tissue series), a disector grid was overlaid on the image with a spacing of 300 µm at 2X (PRH and ACC) and 200 µm at 4X (BLA) magnification using a Nikon Eclipse 80i microscope and 1600x1200 megapixel digital camera connected to a Dell Precision computer (T3500). The ACC was operationally defined according to the definition given by Vogt and Paxinos (2012) and included areas IL, PrL, Cg1, and Cg2 in ranging from 5 mm anterior to bregma to 0 mm in the Vogt and Paxinos (2012) atlas. The PRH included areas PRH and ECT ranging from -3.0 mm anterior to bregma to -6.72 mm and the BLA ranged from -2.04 mm anterior to bregma to -4.08 mm. For each disector contacting the reference space, the number of cells expressing c-fos within a 3450 μm^2 optical fractionator were counted at 100x magnification. Despite the section thickness averaging 39.60 µm (SD = 0.11) in the ACC, 39.70 µm (SD = 0.40) in the PRH and 39.63 µm (SD = 0.71) in the BLA at the time of quantification, only cells within the middle 15 µm of the tissue were counted. This provided a guard height greater than 5 µm in all sections to avoid quantifying near the cut surfaces of the sections where cells may be cleaved/removed by the blade of the cryostat. These parameters were used to ensure that at least 200-300 objects in the ACC and 100-200 in the PRH and BLA would be counted for each brain, which has been shown to be an ideal number of counted objects to obtain accurate and reliable estimates within a reference space (Mouton

2002). Figure 3.1 illustrates representative c-fos positive cells that would have been quantified if within a disector. Finally, the number of cells counted was multiplied by the inverse of 1) the respective section sampling fraction, 2) the area sampling fraction, and 3) the thickness sampling fraction to obtain the estimate of the total number of c-fos-positive cells in each area.

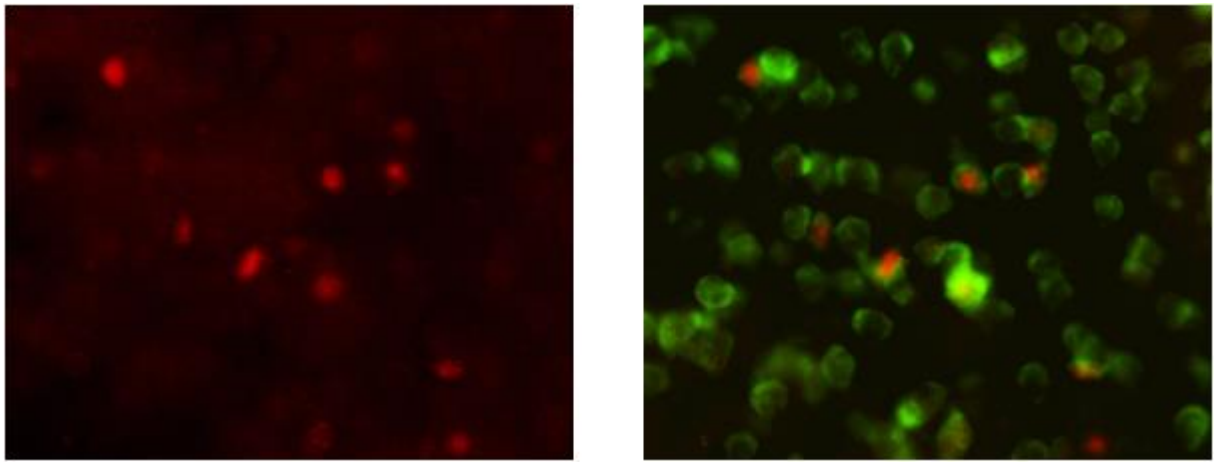


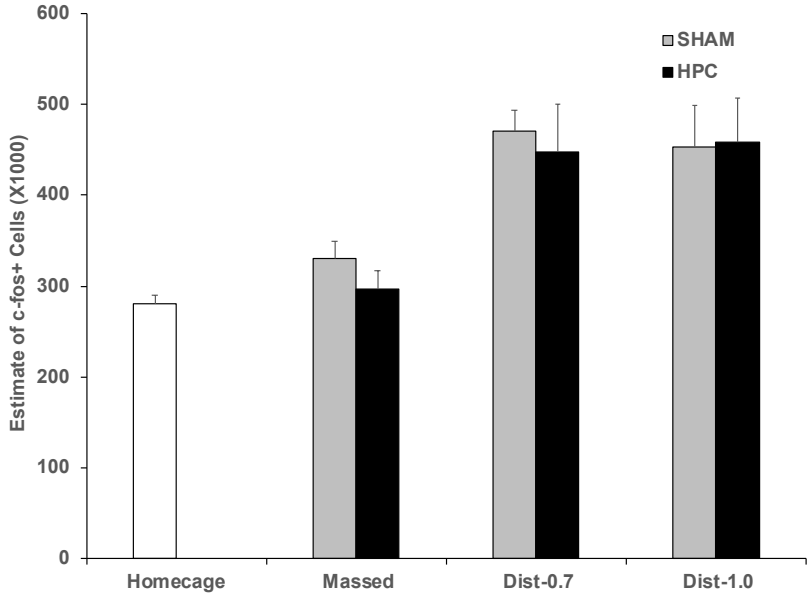
Figure 3.1. On the left, a photomicrograph (60X) showing c-fos positive cells (bright red spheres) in the ACC. On the right, the same c-fos positive cells are co-labeled with Nissl (green), a marker for neurons.

3.3 Results

The estimates of c-fos positive cells in the ACC, PRH and BLA are illustrated in Figures 3.2, 3.3, and 3.4 respectively. These figures also depict the relative estimate of c-fos positive cells for each experimental group in relation to the Homecage control group. This transformation was conducted to ease the statistical analysis for the factorial design. For each area, a 2 X 3 between-subjects ANOVA with Lesion (Sham and HPC) and Conditioning (Massed, Distributed-0.7 mA, and Distributed-1.0 mA) as factors were conducted on relative percent c-fos expression estimates.

ANTERIOR CINGULATE CORTEX C-FOS ESTIMATES

A)



B)

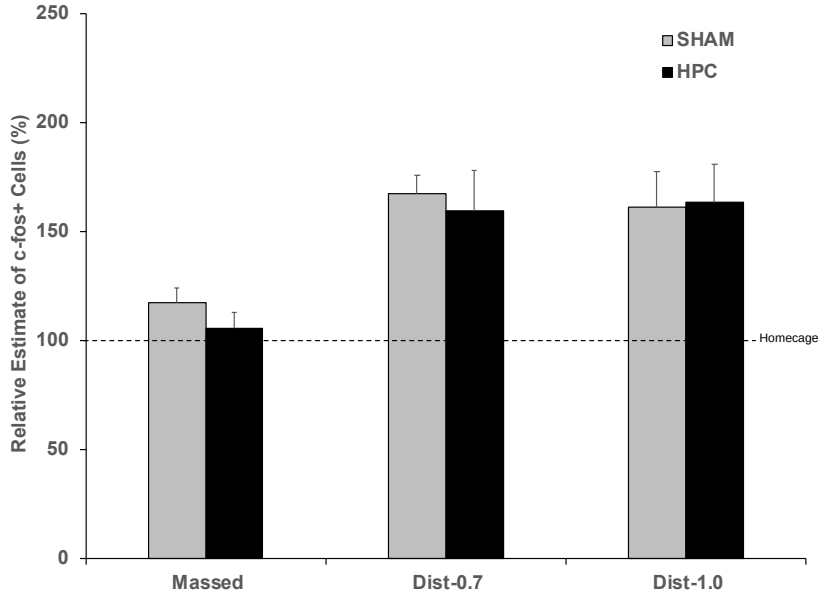
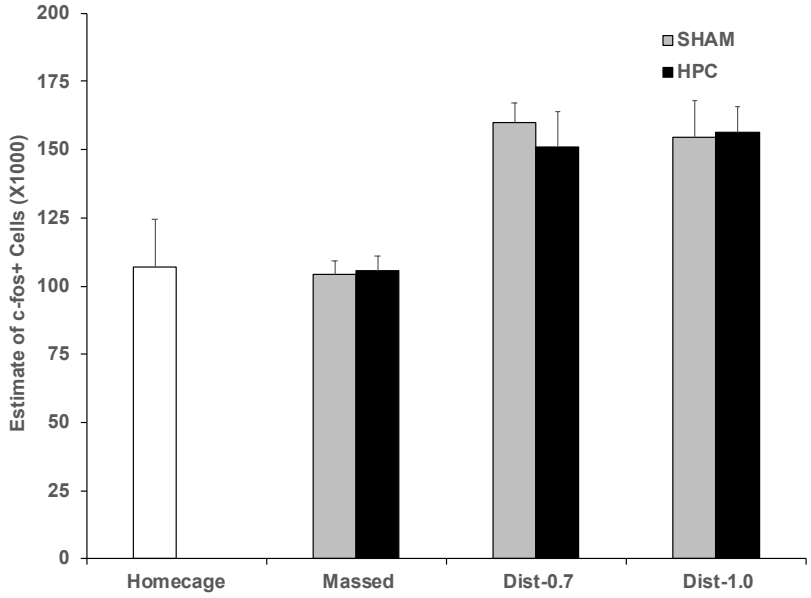


Figure 3.2. A) Mean (+SEM) estimated number of c-fos positive cells in the ACC for the Homecage control and the experimental groups. B) Mean (+SEM) relative (normalized to Homecage) number of c-fos positive cells in the ACC for each experimental group. The Distributed conditions resulted in more c-fos expression than the Massed condition ($p < 0.05$) and all the groups from the Distributed condition showed significantly more c-fos expression than baseline (Homecage; $p < 0.05$). Thus, the ACC is recruited on the retention test following distributed context fear conditioning, even in amnesic cases (HPC-Dist-0.7 mA).

PERIRHINAL CORTEX C-FOS ESTIMATES

A)



B)

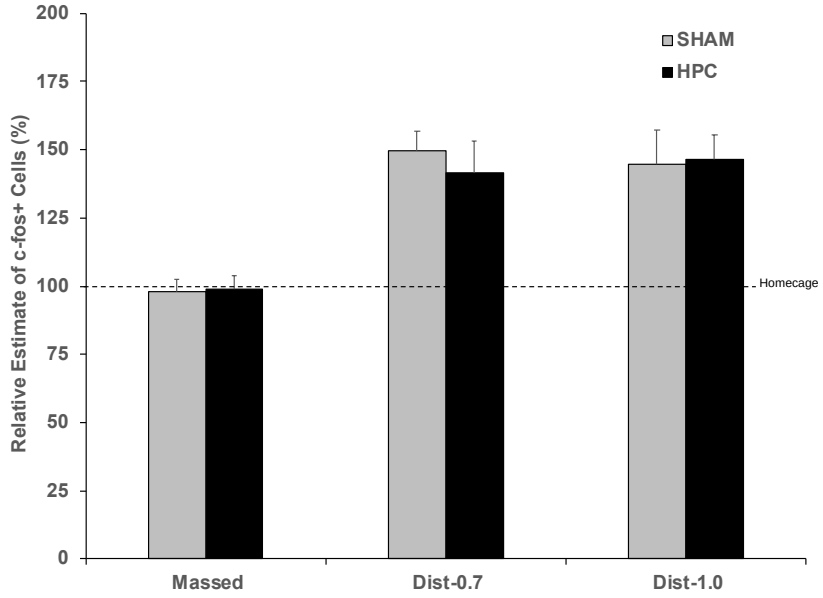
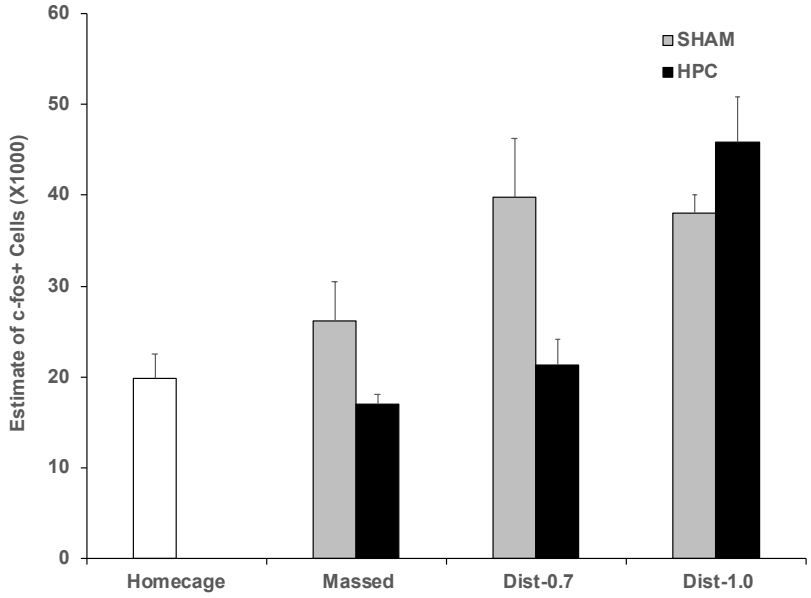


Figure 3.3. A) Mean (+SEM) estimated number of c-fos positive cells in the PRH for the Homecage control and the experimental groups. B) Mean (+SEM) relative (normalized to Homecage) number of c-fos positive cells in the PRH for each experimental group. The Distributed conditions resulted in more c-fos expression than the Massed condition ($p < 0.05$) and all the groups from the Distributed condition showed significantly more c-fos expression than baseline (Homecage; $p < 0.05$). Thus, the PRH is recruited on the retention test following distributed context fear conditioning, even in amnesic cases (HPC-Dist-0.7 mA).

BASOLATERAL AMYGDALA C-FOS ESTIMATES

A)



B)

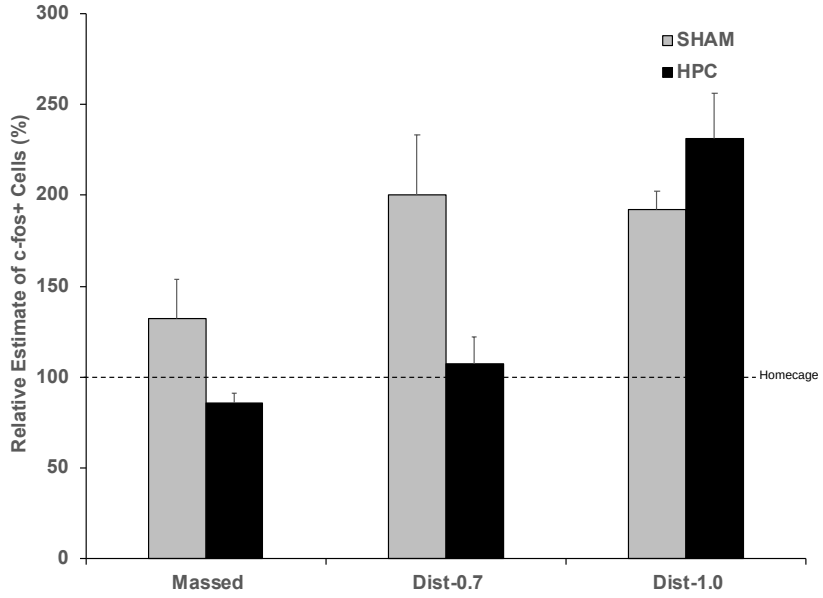


Figure 3.4. A) Mean (+SEM) estimated number of c-fos positive cells in the BLA for the Homecage control and the experimental groups. B) Mean (+SEM) relative (normalized to Homecage) number of c-fos positive cells in the BLA for each experimental group. The Sham-Dist groups showed greater c-fos expression than baseline (Homecage) and the Sham-Massed group (ps < 0.05). The HPC-Dist-1.0 mA group, which showed strong memory on the retention test, had significantly more c-fos expression than baseline (Homecage) and the other two HPC groups that suffered from retrograde amnesia (ps < 0.05), as well as comparable estimates to its respective Sham group (p = 0.145). Thus, the BLA is recruited on the retention test following distributed context fear conditioning, but only when the context fear is expressed (no amnesia).

The analysis of the relative c-fos positive cells estimates in the ACC (Figure 3.2) indicated a significant main effect of Conditioning, $F(2, 35) = 9.201$, $p < 0.05$. The main effect of Lesion, however, was not significant, $F(1, 35) = 0.262$, $p = 0.612$, nor was the interaction, $F(2, 35) = .143$, $p = 0.867$. Pairwise comparisons on the Conditioning main effect revealed that both the Dist-0.7 mA and Dist-1.0 mA groups had significantly more c-fos positive cells than the Massed group (ps < 0.05). In contrast, the Dist-0.7 mA and Dist-1.0 mA groups had comparable c-fos estimates ($p = 0.928$). One-sample t tests (vs. 100%) were also conducted to determine whether the estimates were greater than Homecage/baseline expression levels. The four distributed groups and the Sham-Massed group showed c-fos estimates significantly above baseline (ps <0.05), whereas the HPC-Massed group did not ($p = 0.452$).

A similar pattern of results was found in the PRH (Figure 3.3). Indeed, the ANOVA indicated a significant main effect of Conditioning, $F(2, 35) = 19.125$, $p < 0.05$. And again, the main effect of Lesion was not significant, $F(1, 35) = 0.06$, $p = .809$, nor was the interaction, $F(2, 35) = 0.19$, $p = 0.828$. Pairwise comparisons on the Conditioning main effect revealed that both the Dist-0.7 mA and Dist-1.0 mA groups had significantly more c-fos positive cells than the Massed group (ps < 0.05). In contrast, the Dist-0.7 mA and Dist-1.0 mA groups comparable c-fos estimates ($p = 0.989$). One-sample t tests (vs. 100%) were also conducted to determine whether the estimates were greater than Homecage/baseline expression levels. The four distributed groups showed c-fos estimates significantly above baseline (ps < 0.05), but not and the Sham-Massed group ($p = 0.652$) nor the HPC-Massed group ($p = 0.842$).

The c-fos data in the BLA showed a pattern of results influenced by the HPC lesions, a least in part (Figure 3.4). In this case the ANOVA indicated that the Conditioning by Lesion

interaction was significant, $F(2, 35) = 6.082$, $p < 0.05$. Further analysis of the interaction with LSD pairwise comparisons revealed that both Sham-Dist groups had significantly higher c-fos expression than the Sham-Massed group (ps < 0.05), but that they did not statistically differ from each other ($p = 0.777$). Interestingly, the HPC-Dist-1.0 mA, which showed good retention performance on the test, had comparable c-fos expression in the BLA to its respective Sham group ($p = 0.145$). Moreover, the HPC-Dist-1.0 had significantly more c-fos expression than the two other HPC groups (ps < 0.05). In contrast, the HPC-Dist-0.7 mA, which suffered from retrograde amnesia, had significantly lower c-fos expression in the BLA as its respective Sham group ($p < 0.05$) and no more than the HPC-Massed that also suffered from retrograde amnesia ($p = 0.385$). The HPC- and Sham-Massed did not significantly differ ($p = 0.081$). Additionally, the main effect of Conditioning was significant, $F(2, 35) = 15.581$, $p < 0.05$, as was the main effect of Lesion, $F(1, 35) = 4.589$, $p < 0.05$. These were, however, not further analyzed because of the prior interaction breakdown. Note that the one-sample t tests (vs. 100%) to determine whether the estimates were greater than Homecage/baseline expression levels confirmed the interaction analysis. Indeed, only the Sham-Dist-0.7 mA, Sham-Dist-1.0 mA and the HPC-Dist-1.0 mA showed c-fos estimates greater than baseline (ps < 0.05). In contrast, the Sham-Massed ($p = 0.197$) and HPC-Dist-07 mA ($p = 0.630$), whereas the HPC-Massed had significantly lower levels ($p < 0.05$).

When conducting unbiased stereology, a coefficient or error is also obtained with the count estimates. This coefficient represents a measure of quantification error (biological and experimental) which is dependent on the degree of sampling during the quantification (Mouton 2002). It enables an inference on how “good” an estimate is and, as a rule of thumb, coefficients of error should be below 10 % (0.10) in order to obtain accurate estimates (Mouton 2002). This,

however, depends on the size of the difference between groups. With larger differences, then larger coefficients of error are acceptable (e.g., 5%) and, vice versa, with smaller differences, then smaller coefficients of error are required (e.g., 5%). The coefficients of error associated with all the group estimates described above are in Table 3.1. No statistical analyses beyond the group means were conducted because the coefficients generally respected the below 10% criteria. Moreover, in the instances in which the coefficients were greater than 10%, differences were still found and/or differences were minimal and would not have benefitted from more sampling to reduce the coefficients.

Table 3.1 Mean coefficient of error for c-fos positive cells in each group per structure

Mean coefficient of error for c-fos-positive cells estimates in each group

	Anterior cingulate cortex		Perirhinal Cortex		Amygdala	
Group	Sham	HPC	Sham	HPC	Sham	HPC
0.7 mA	0.05	0.12	0.05	0.08	0.16	0.14
1.0 mA	0.10	0.11	0.09	0.06	0.05	0.12
Massed	0.06	0.07	0.05	0.05	0.16	0.06
Homecage Controls	0.03		0.16		0.14	

3.4 Discussion

Highly salient distributed contextual fear learning episodes can resist damage to the HPC. This experiment aimed to identify the non-HPC structures that come to support these memories. High ACC and PRH c-fos levels were present in the rats from the Dist-1.0 mA and Dist-0.7 mA

regardless whether the rats received HPC lesion or not, when compared the Massed or Homcage controls group. Sham BLA c-fos level was high in the Dist-0.7 mA and the Dist-1.0 mA when compared to the levels for the rats in the Massed group and Homecage controls. Importantly, there was high level of c-fos activation in the BLA for HPC lesion rats in the Dist-1.0 mA condition when compared to HPC-lesion rats in the Dist-0.7 mA and Massed conditions.

These findings suggest that the PRH and ACC are recruited to support the non-emotional features of the memory that became strong over reinstatements. Moreover, these finding imply that the PRH and ACC are part of the non-HPC network that comes to support distributed contextual aspect of the memory. Additionally, these findings suggest that the BLA is recruited during successful expression of a highly salient context fear memory that has become HPC independent over reinstatements. Based on this finding, clearly a non-HPC network is supporting the memory. Together the ACC, PRH and BLA forms a network that supports distributed highly salient condition context fear episodes.

CHAPTER 4 - GENERAL DISCUSSION

Distributing context fear conditioning sessions rapidly creates a memory that can be expressed with disruption to the HPC (Lehmann et al., 2009; Lehmann, & Mcnamara, 2011). This thesis examined whether the distribution of the conditioning episodes alone is sufficient or whether a combination of distribution and high conditioning saliency is necessary to allow a context fear memory to resist damage to the HPC. Additionally, this thesis explored the non-HPC system that comes to support the spared memory. Specifically, the ACC, PRH and BLA were investigated as possible structures that support reinstated context fear memories.

In fact, the results suggested that independently, distribution or high conditioning saliency alone of a context fear memory is insufficient to resist damage to the HPC. But, together distribution and highly salient conditioning results in spared memory. There is indeed a non-HPC network that comes to support the memory that survives HPC damage. Quantification of c-fos expression in the ACC, PRH and BLA illustrated that the ACC and PRH supports the context representation of the memory while the BLA supports the fear representation. Together the ACC, PRH and BLA form a network that functions effectively to support a highly salient distributed context fear memory.

4.1 The Role of Saliency in Forming a Memory that Survives Damage to the HPC

In chapter 2, we examined the role of saliency in making a HPC-dependent context fear memory survive damage to the HPC. We found that a distributed low salient event does not provide effective conditioning since rats did not show evidence of context fear memory when comparing the first and last conditioning sessions. The rats in the distributed intermediate saliency condition displayed robust conditioning during acquisition but RA following HPC damage when compared to its respective control group. More importantly, the rats in the

distributed high saliency condition showed strong conditioning during acquisition and intact memory following HPC-damage, comparable to controls. These findings suggest that distribution of the event alone is insufficient in creating a spared context fear memory. The conditioning event needs to be highly salient and distributed to mitigate RA following HPC damage.

4.2 Distributed Reinstatement Theory

The findings of these experiments introduce an aspect of the DRT that was previously overlooked. Following HPC lesion, 10 high salient shocks given in a single learning session result in RA. However, if the same number of high salient shocks is distributed over five consecutive days the memory mitigates RA. This suggests that the learning event not only needs to be distributed but it also needs to be a highly salient experience for it to resist damage to the HPC. Saliency does in fact, play a crucial role in mitigating RA after HPC damage following the distributed learning episodes.

4.3 Structures that Support Highly Salient Context Fear Memories

The experiments in Chapter 3 examined whether the ACC, PRH or BLA play a role in supporting the highly salient distributed context fear memory. Chapter three used quantification of c-fos, a protein that is upregulated following neuronal stimulation, to determine whether the ACC, PRH or BLA come to support the spared context fear memory.

The results suggest that the BLA supports the fear representation of the distributed highly salient context fear memory. C-fos activation in the BLA was highest in the HPC-lesion rats that were in the Dist-1.0 mA condition (group with spared memory following HPC lesion) suggesting that the BLA supported the fear aspect of the distributed highly salient memory. The ACC and PRH supports the context representation of the memory, as rats in the Dist-0.7 mA and Dist-1.0

mA conditions, regardless of surgery type, displayed high c-fos activation levels when compared to the c-fos activation for rats in the Massed condition and Homecage controls.

Eacott & Gaffan (2005) explains that despite strong connections between cortical structures like the ACC and PRH with the HPC, the HPC has a distinct function of its own. It is responsible for combining information about contexts, objects and position (Eacott & Gaffan, 2005). In the same manner, based on the results of our experiments, it appears when the HPC is intact it has a similar role of combining information about context and fear; hence sham rats displayed the ability to remember the context associated with the foot-shock. When the HPC is damaged, the ACC, PRH and BLA remain part of the network that support parts of the highly salient distributed context fear memory. The ACC and PRH support the context representation while the BLA supports the fear representation of the memory and together these structures support the memory with damage to the HPC. Hence, HPC-lesion rats in the high salient condition are still able to express the contextual fear memory.

4.4 The Role of the ACC, PRH and BLA in Supporting Context Fear Memory

As mentioned previously, the BLA plays a critical role in both the acquisition and expression of conditional fear (Gale et al., 2004). Specifically the BLA is associated with fear processing (Maren & Fanselow, 1996). The findings of the Experiments in Chapter 3 support this view. Moreover, the PRH was previously implicated in processing of context representation of context fear memory where neurotoxic lesions of the PRH resulted in anterograde and retrograde deficits in conditioning to the training context in an unsignaled shock paradigm (Buccia, Phillips, & Burwell, 2000). The findings of the experiments conducted here are in support that the PRH supports the contextual representation of the context fear memory as rats in the distributed conditions, regardless of type of surgery displayed high level of c-fos activation in the PRH

when compared to the Homecage controls or Massed group. Contrastingly, the finding that the ACC also supports the context representation of the memory is different from previous findings (Tang et al., 2005). The ACC is known to be involved in context and fear memory, but we found that the structure is actually involved in the context representation of the distributed contextual fear.

4.5 Generalized c-fos Expression?

It can be argued that c-fos expression is enhanced in the whole brain and it is not just in the ACC, PRH or BLA. To confirm that this is or is not the case a follow up experiment could be performed where c-fos expression in a structure not associated in any way with context fear memory would be assessed. Good structures to look at would be the Granular Insula or Somatosensory Area 2, since they are usually not implicated in supporting context fear memories (Schneider, Friedman, & Mishkin, 1993; Yau, Connor, & Hsiao, 2013). If c-fos expression is low in either of these structures then we can definitively conclude that the ACC, PRH and BLA are indeed supporting the spared context fear memory.

4.6 The SMSC and MTT

Generally, memories acquired recently, say yesterday for instance, is likely to be remembered compared to memories acquired 3 years ago (remote memory). These experiments conducted here find with damage to the HPC, if the experience is highly salient and distributed, memories acquired quite recently mitigate RA. This finding contradicts the explanations given by the system consolidation theories. Our results actually suggest a reverse temporal gradient as opposed to a loss of recent and sparing of remote memories, which is the pattern supported by the SMSC (Marr, 1971) and the MTT (Nadel & Moscovitch, 1997). So, the finding that recently

acquired highly salient distributed memory mitigates RA following HPC damage contradicts the system consolidation theories, thereby adding new information and an advancement in the explanation of the way context fear memory are consolidated.

4.7 Beyond the Behavioral Finding

It is possible that the rats in the Dist-1 mA condition remember and the Dist-0.7 mA doesn't because the high salient shock was strong enough to trigger protein synthesis, LPT or neurogenesis. Keep in mind that this was not explored in this thesis but it is a possible explanation why the rats in the Dist-1 mA condition remembered while the Dist-0.7 mA did not. The finding of this thesis supports that strong foot shocks distributed over several sessions created a memory that is stronger in the entire memory network that supports the memory. So, if one component of the network, the HPC in this instance, is damaged, the rats can still express the memory because the other structures in the network have enough of a representation to support it.

To examine whether the HPC disengages in supporting the distributed highly salient experience, c-fos expression of the HPC in sham rats in the Dist-1 mA condition could be assessed. If c-fos level is high then it can be concluded that the HPC does not become disengaged but rather remain involved in supporting the memory. If c-fos expression is low in the HPC then it can be concluded that the HPC disengages and does not play a role in supporting these memories. We predict that the HPC will have high c-fos expression, meaning the distributed high salient memories does not switch dependence from one structure to the next but rather creates a network that works together to support the memories.

4.8 Massed Condition and RA

Newly acquired memory is initially in a fragile state and can be disrupted by several types of interference (Robertson, 2012). Over time, the memory becomes resilient to this interference through a process known as consolidation (Robertson, 2012). HPC-lesion rats in the massed condition displayed RA. This is perhaps because the memory only had one bout of cellular consolidation where there was one increment of the memory consolidated in the neocortex. There was only one episode to consolidate while in the distributed condition there were 10 episodes to consolidate. Ten bouts of cellular consolidation led to a more represented memory in the ACC, PRH and BLA.

4.10 Cellular Consolidation and Mr. Bob

Mr. Bob who was introduced in the introduction told an epidemiology about where the word shit originated. Based on the results of these experiments, it can be inferred that he remembered this story because he'd told it so many times. On a cellular level, every time to told the story or every time he heard the word shit and thought about the story there was a representation of that memory that was placed in his neocortex. So, when his HPC was damaged, he remembered the story because there was enough of a representation present to support it.

4.11 Is Saliency Necessary for Other Memories?

It is important to realize that the findings of this thesis might be applicable (with a grain of salt) to other types of memory that is not a context fear memory. Patient T.T. who was previously mentioned in the introduction, had bilateral HPC lesions but could remember London taxi routes he frequently used before his HPC damage. He performed as well as control participants in navigating the streets of London (Maguire et al. 2006). It is possible that Patient T.T. remembered these routes because he used them many times, hence they were reinstated and became more resistant to HPC damage. It is also possible that they were highly salient events.

Patient T.T. spent majority of his day navigating these routes, and it is possible that encounters with passengers or other drivers on the roadway made some of his encounters salient. Hence even with bilateral HPC lesion, Patient T.T. memories of these routes were spared.

4.12 Conclusion

The current thesis aimed at examining whether any memory distributed over several learning sessions will result in spared context fear memory or whether high saliency and distribution is necessary to allow the memory to survive HPC damage. Experiment 1 found that high saliency and distribution of the conditioning episodes is necessary to create a memory that resists damage to the HPC. Chapter 2 explored the whether the ACC, PRH and BLA are structures that support these memories. This was done by examining the c-fos expression levels following the retention test in experiment 1. C-fos expression was highest in the BLA of the HPC-lesion rats in the Dist-1.0 mA condition, thereby suggesting that the BLA supports the fear representation of the memory. In contrast, there was high levels of c-fos activation in the ACC and PRH for rats in distributed conditions (Dist-0.7 and Dist-1.0 mA) when compared to those in the Massed condition and Homecage controls. This suggests that the ACC and the PRH supports the context representation of the memory. Together, the ACC, PRH and BLA support the expression of the of the context fear memory without an intact HPC.

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