Temporally-graded retrograde amnesia for cocaine place preference memories following hippocampal lesions

by

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Abstract

Memory is thought to be a dynamic process, as its strength, quality and anatomical organization change as a function of time. The systems consolidation hypothesis of memory postulates that while some memories are initially dependent on hippocampus, they gradually become immune to hippocampal disruptions over time. Although this temporal gradient has been observed in many types of memories, such as contextual fear memories, little is known about the reorganization of contextual drug-associated memory. In the present study, we examined the role of hippocampus in cocaine conditioned place preference (CPP) memory at different time points after conditioning. First, we found that excitotoxic hippocampal lesions impaired recent (1 day), but not remote (30 days) cocaine CPP memory in mice, suggesting that contextual drug-associated memory reorganizes over time, and becomes independent of the hippocampus. However, this remote hippocampus independent memory was disrupted if a reminder (drug+context) was administered one day before surgery, suggesting that a reminder rendered this older memory dependent on the hippocampus. Third, overexpressing myocyte enhancer factor 2 (MEF2), a transcription factor that disrupts structural plasticity, in hippocampus blocked cocaine CPP association, suggesting that structural changes in hippocampus is required to form a contextual drug-associated memory. Together, these studies are a first step in examining whether and how

context-drug memories reorganized over time. These results may be particularly important as context-drug associations are implicated in drug relapse.

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AAV: adeno-associated virus

AMPA: 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid

CaMKII: calcium/calmodulin-dependent protein kinase II

CAMP: adenosine 3',5'-cyclic monophosphate

CBP: CREB binding protein

CMV: cytomegalovirus immediate-early gene

CPA: conditioned place aversion

CPP: conditioned place preference

CREB: cAMP response element-binding

CRTC1: CREB-regulated transcription coactivator 1, or TORC1 (Transducer Of Regulated CREB activity 1)

CS: conditioned stimulus

CXT: context

D1R: dopamine receptor 1

DAPI: 4',6'-diamidino-2-phenylindole

DNQX: glutamate antagonist 6,7-dinitroquinoxaline-2,3-dione

DG: dentate gyrus

E-LTP: early phase LTP

EPSP: excitatory postsynaptic potential

ERK: extracellular signal-regulated kinase

HSV: herpes simplex virus

GABA-A: γ-aminobutyric acid

GFP: green fluorescent protein

IEG: immediate early gene

ISI: interstimulus interval

ITI: intertrial interval

L-LTP: late phase LTP

LTD: long-term depression

LTP: long-term potentiation

MAPK: mitogen-activated protein kinase

MEF2: myocyte enhancer factor 2

NAc: Nucleus accumbens

NMDA: N-methyl-D-aspartic acid

PBS: phosphate-buffered saline

PFA: paraformaldehyde

PKA: protein kinase A

PKC: protein kinase C

PTSD: posttraumatic stress disorder

SNc: medial substantia nigra pars compacta

US: unconditioned stimulus

VTA: ventral tegmental area

1 Literature Review

1.1 Dynamics of drug-associated memories

Memory storage can be viewed as a process in which we receive, organize, store, and retrieve all the information that we encounter every day. Through memories, we define who we are and connect ourselves to our past experiences and knowledge, so that we can react to new environmental stimuli and information. It has been suggested that memories may not be static. Rather, memories can be thought of as dynamic and changing in their organization, strength, and even quality as a function of time.

In the experimental animal literature, the strongest evidence for time-dependent changes in organization comes from studies of fear-related memories. In fear conditioning paradigms, a neutral context or cue is associated with an aversive event such as a footshock. Lesion studies have shown that the brain structures supporting these types of memories changes as a function of time. Other types of associative memories, including drug-associated memories may also be similarly dynamic. For instance, even during abstinence, drug cravings elicited by previous drug-associated context increases over time, suggesting that these memories do, in fact, change over time. Although the reorganization of drug-associated memories has not been systematically studied, the time-dependent reorganization of various types of memory has been previously described. For example, the recall of recent spatial memories and recent contextual fear memories involves activation of the hippocampus, whereas recall of the remote spatial and contextual fear memories mainly involves activation of cortical regions (Frankland and Bontempi, 2005). In the current study, we examined whether the hippocampal dependency of drug-associated contextual memories changes as a function of time. With the framework of systems consolidation of contextual fear memories, we used cocaine-conditioned place preference (CPP) to investigate how the circuits supporting drug-associated contextual memories develop over time.

1.2 Systems consolidation of memory

1.2.1 Historical review of discovering memory traces

1.2.1.1 Search for the engram: Lashley and Hebb's theories

Memories connect us to our past experiences and define who we are today. Memory is not only the ability to acquire, store, and retrieve information, but is also essential for organisms to adapt and survive. Amnesia and pathological long-lasting memories can both be devastating, as they profoundly impact our daily life. Because of the importance and the enigmatic nature of memory, much research has been done in the past century in this field, with a focus on what brain regions are responsible for memory. However, the molecular process of memory and the roles of particular brain regions in specific types of memories are still not clear, even though they have received much attention in research.

Many brain functions begun to be mapped in the nineteenth century. For example, the specific brain regions responsible for sensory functions, motor control, and language (Broca, 1861) were identified. However, the actual storage sites for our memories were difficult to define. In the early twentieth century, German researcher Richard Semon introduced the influential term "engram" to describe the neuronal substrates that respond to external stimuli, store, and express memory. In his book "The Mneme," Semon proposed that stimuli produce a "permanent record, …written or engraved on the irritable substance," or the "engram" (Semon, 1921, p.24). Consistent with the current view, Semon also thinks that this "engram" would be retrieved again when the elements resembling components of the original context were encountered. However, Semon did not know where exactly the engram was located in the brain.

Later in the twentieth century, researcher Karl Lashley (1929) suggested that the engram could not be localized to one area in the brain, but was rather distributed across the brain. The process of localizing the engram in the brain largely relied on lesioning technique in animal models and on the study of brain-damaged patients. One of the most well-known lesion studies was published by Lashley in 1929. In this series of studies, Lashley trained rats in various memory tasks, such as food-rewarded mazes, and then lesioned various percentages of the cortex. Unfortunately, he did not find a specific location that was especially critical for the memory of the maze. The same result was found even when the lesions were performed before training.

After 30 years of work, he summarized his observations in the paper entitled “In Search of the Engram,” in which he concluded that cortical lesions impaired rats’ performance in the maze and that the degree of impairment was roughly correlated to the amount, but not to the location, of the lesion. This observation led to the hypothesis that memory must then be distributed throughout the brain. He later proposed two principles: (1) the principle of mass action, whereby the cortex acts as a whole and (2) the principle of equipotentiality, meaning that if parts of the cortex are damaged, other parts may take on the role of the damaged portion for learning (Josselyn, 2010; Lashley, 1929).

Donald Hebb (1949) later proposed the cell assembly theory, suggesting that Lashley’s results could be interpreted as a coordinated representation of distributed cells in the cortex, with different areas storing different features of the whole representation (Hebb, 1949). In his influential book, the “Organization of Behavior” Hebb proposed that these assemblies of cells responding to stimuli might connect together during learning and become the engram. In this book, he proposed a general principle that can be referred as the Hebbian theory, or the cell assembly theory: “Let us assume that the persistence or repetition of a reverberatory activity (or "trace") tends to induce lasting cellular changes that add to its stability… When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.”

Why did Lashley not found evidence for an engram? It is possible that the memory task was too complicated to rely on only one brain region, or simply that other brain regions, instead of the cortex, are involved in memory. After Lashley’s research on animal models, researchers continued to attempt the localization of the engram by studying brain-damaged patients and attention shifted to the hippocampus (Josselyn, 2010).

1.2.1.2 H.M. and multiple memory systems

It was not until the 1950s that Brenda Milner, who described the amnesia in the most well-known case patient H.M., that the medial temporal lobe as successfully identified as a critical part of the “engram” (Scoville & Milner, 1957). Patient H.M. had parts of his medial temporal lobe removed to treat intractable epilepsy (Scoville & Milner, 1957). This surgery successfully reduced the frequency of H.M.’s seizures with no indication of impairment in personality or

intelligence. However, following this procedure, H.M. suffered severe amnesia, both in the anterograde and the retrograde direction. H.M.'s ability to form new memories was profoundly impaired (anterograde amnesia); he could not form memories of events or acquire new general knowledge normally. H.M. also suffered from retrograde amnesia: he lost his past memories. However, unlike the complete anterograde amnesia, this retrograde memory loss was temporally graded. That is, although he lost more recent memories, he retained memories from his early childhood (Scoville & Milner, 1957). Further analyses revealed that H.M.'s retrograde amnesia extended back approximately 11 years (Sagar et al., 1985). With H.M's case and other human studies, it has been proposed that the medial temporal lobe plays a time-limited role in memory processes (Squire, 2004). There are also other brain regions implicated in memory. For example, Victor (1971) showed that damage to the thalamus, which is often caused by Korsakoff's syndrome in chronic alcoholics, leads to impairment in memory (Victor, 1971).

The idea that different brain regions support different types of memories also emerged from the observations of H.M. Although H.M. suffered severe memory loss and was not able to form new explicit memories, he retained the ability to acquire procedural memory through mirror drawing, a task which requires hand-eye coordination skills (Milner, 1972). Interestingly, H.M. could retain this memory for up to a year (Gabrieli, 1993). In another set of studies, amnesic patients acquired the skill of reading mirror-reversed words, which requires perceptual and cognitive skills in addition to motor skills, while they also exhibited intact priming effects (Cohen & Squire, 1980). Along with other studies looking at the perceptual learning and priming memory, these data led scientists think that memory itself is not a unitary phenomenon. As certain patterns of brain damage often associated with certain patterns of memory deficits, these studies suggested that there are several anatomically distinct memory systems (Gaffan, 1974; Hirsh, 1974; McKee & Squire, 1992).

Based on these studies, multiple memory systems have been proposed to explain why memory is not a unitary, homogenous process. Cohen and Squire (1980) proposed that memory can be divided into two broad categories: declarative and non declarative (Cohen and Squire, 1980). Declarative memory is conscious memory, which describes the recollection of facts and events, and depends on the medial temporal lobe. On the other hand, non declarative memory is unconscious memory, which is expressed through performance rather than recollection, and includes procedural learning, priming and perceptual learning, and classical conditioning

(Squire, 1987). Using this taxonomy, declarative memory can be further classified into semantic and episodic memory. Tulving (1972) described that semantic memory was about the fact, and episodic memory was about the event (Tulving, 1972). That is, episodic memory is associated with the space and time of its acquisition, while semantic memory refers to general knowledge of facts. For example, patient H.M. lost the ability to form new declarative memories, suggesting that he could not form lasting memories of events (episodic memory) or acquire new general knowledge and facts normally (semantic memory).

Multiple memory systems have also been demonstrated in animal models. For example, O'Keefe and Nadel (1978) proposed that the hippocampus is essential in a specific type of memory in their influential book "The Hippocampus as a Cognitive Map." They stated in the book: "The hippocampus is the core of a neural memory system providing an objective spatial framework within which the items and events of an organism's experience are located and interrelated" (O'Keefe & Nadel, 1978). In the lesion study done by White and McDonald (1993), they demonstrated the different roles of the hippocampus, amygdala, and caudate nucleus in different memories. It was showed that the hippocampus was necessary remembering the relationships among multiple cues, whereas the amygdala was necessary for the task in which cues were associated with reinforcers. Lastly, the caudate nucleus was necessary for figuring out that an individual cue was associated with the correct response (White & McDonald, 1993).

Studies in patients and in animal models have shown similar trends in identifying brain regions for declarative memory. Research on brain damaged patients, such as H.M., has shown that declarative memory largely depends on the medial temporal lobe (including the hippocampus). In animal models, contextual memory (memory about the configuration or interrelation of multiple cues in a given context) largely depends on the hippocampus. However, it is also noted that in the multiple memory systems theory, these systems do not necessarily act independently; they can act in parallel when learning a task (White & McDonald, 2002; Squire, 2004).

1.2.2 The consolidation of memory

1.2.2.1 An overview

The term "consolidirung" (consolidation) of memory is thought to first be introduced by Müller and Pilzecker. Müller and his student Pilzecker proposed the idea of memory consolidation in

their seminal monograph “Experimentelle Beiträge zur Lehre vom Gedächtnis” (Experimental Contributions to the Science of Memory), published in 1900. Basically their idea was that learning itself does not induce a permanent memory, but that the memories need to be consolidated and it takes time. That is, a recently-acquired memory may gradually transform from an a labile state to a more permanent state (Lechner, Squire, & Byrne, 1999; Frankland & Bontempi, 2005).

Memory consolidation is generally thought to involve two processes that occur on different timescales and at different levels of the brain: synaptic consolidation and systems consolidation. Synaptic consolidation refers to the molecular and cellular processes that occur within hours of memory formation to stabilize changes in synaptic connectivity in local circuits (such as the growth of new synaptic connections and the restructuring of existing ones). Synaptic consolidation happens within milliseconds of the stimulus; responding neurons in certain brain regions have their channels open and calcium enters the cell. Within seconds, the second messenger systems are activated, and within minutes this leads to the activation of transcription factors in the cell. However, after memory formation, it has been observed that for a short time period the memory is vulnerable to disruptions, including distracters, drugs, or seizures. In order to “consolidate” the memory, the activation of transcription factors leads to the modulation of gene expression and the synthesis of new proteins to strengthen and form new synaptic connections. In contrast to synaptic consolidation, system consolidation is a more prolonged process and occurs at a system level. It refers to the gradual reorganization of the brain regions that support memory and occurs in the weeks, months, and even years after memory formation (Dudai, 2004; Frankland & Bontempi, 2005; Josselyn, 2010).

1.2.2.2 Synaptic consolidation

Molecular processes

Some of the first studies investigating the molecules involved in synaptic consolidation have been conducted in Drosophila (flies) and Aplysia (sea slugs). Random genetic mutations were introduced in the Drosophila, and thousands of mutants were screened in a behavioral assay. In this assay, the Drosophila formed a simple association between an odor and a shock. When this memory was tested in all the mutants, many genes interfering with the memory were identified. Most of them were involved in the adenosine 3',5'-cyclic monophosphate (cAMP) second

messenger pathway (Davis, Schuster, & Goodman, 1996). The same cAMP pathway was also identified in the gill withdrawal reflex sensitization studies the Aplysia (Brunelli, Castellucci, & Kandel, 1976). CREB, the cAMP Response Element-Binding protein, is a critical transcription factor in this pathway; it is activated by the cAMP pathway and leads to protein synthesis for the modification of existing synapses and for the creation of new synaptic connections (Yin & Tully, 1996). This cascade of molecular events leads to changes in synaptic connectivity in neurons and, in turn, consolidates the memory.

Interestingly, studies have shown that molecules such as CREB are highly conserved across species (from Aplysia to Drosophila to mice) and across different memory systems (Lechner, Squire & Byrne, 1999). Firstly, CREB was identified as a critical protein for the transformation of short-term to long-term memory in the Drosophila odor-shock memory model (Yin & Tully, 1995). Secondly, in Aplysia, CREB was demonstrated to play a similar role in the long-lasting facilitation of the gill withdrawal reflex (Dash, Hochner, & Kandel, 1990; Kaang, Kandel, & Grant, 1993). Thirdly, CREB has been implicated in the fear memory in mice (Bourtchuladze et al., 1994). Manipulating CREB not only disrupts the formation of fear memory and increased CREB function facilitates fear memory formation by "driving" the memory into the neurons. Moreover, it has been shown that selectively ablating neurons overexpressing CREB in the lateral amygdala specifically erased the fear memory (Han et al., 2009; Josselyn et al., 2001; Josselyn, 2010). Lastly, in humans, the role of CREB can be implicated from patients with Rubinstein-Taybi syndrome. In this syndrome, patients suffer from cognitive dysfunction caused by a rare genetic mutation in the region of the genome that encodes CREB binding protein (CBP), an important protein interacting with CREB (Petrij et al, 1995).

Cellular process: Long-term potentiation

As memories are thought to be encoded in synapses, long-term potentiation (LTP) is considered the major cellular mechanism that underlies memory (Bliss & Collingridge, 1993; Cooke & Bliss, 2006). First discovered in the rabbit hippocampus by Terje Lømo in 1966, LTP refers to a high-frequency train of stimuli in pre-synaptic fibers that produce a long-lasting enhancement in the postsynaptic cell's response to subsequent single-pulse stimuli (Lømo, 2003). As a result of LTP, a long-lasting "cellular memory" (enhancement in signal transmission) between two neurons can be formed and stored. Another cellular process of memory, long-term depression (LTD), has also been identified. As the opposing process to LTP, LTD is the long-lasting,

activity-dependent reduction in efficacy of signal transmission (Massey & Bashir, 2007). Both LTP and LTD can be induced rapidly, both depend upon protein synthesis, and both can last from hours to months. Therefore, LTP and LTD are widely considered as the cellular mechanisms of long-term memory (Cooke & Bliss, 2006; Milner, Squire, & Kandel, 1998).

The mechanism of LTP can be described in two phases: the early phase LTP (E-LTP), and the late phase LTP (L-LTP). The induction of E-LTP occurs when the concentration of calcium inside a postsynaptic cell exceeds a critical threshold. Initially, glutamate binding to the AMPA (2-amino-3-5-methyl-3-oxo-1,2-oxazol-4-yl propanoic acid) receptor initiates an influx of sodium ions into the postsynaptic cell, producing a short-lived depolarization called the excitatory postsynaptic potential (EPSP). If the magnitude of EPSP is sufficient, E-LTP will be induced. If a single stimulus does not produce enough EPSP, repeated stimuli can trigger the postsynaptic cell to be progressively depolarized, then generate sufficient EPSP to relieve the magnesium blockade of the NMDA receptor, allowing a calcium influx. The rapid rise in intracellular calcium concentration triggers the activation of several enzymes that mediate E-LTP induction, including calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK). The persistent activation of CaMKII and PKC is necessary for the maintenance of this synaptic enhancement (Sweatt, 1999).

Late LTP is the extension of E-LTP and requires gene transcription and protein synthesis in the postsynaptic cell (Frey, Krug, Reymann, & Matthies, 1988; Frey, Müller, & Kuhl, 1996). It is generally thought that the first phase of L-LTP depends upon protein synthesis, while the second L-LTP depends upon both protein synthesis and gene transcription (Lynch, 2004). For example, extracellular signal-regulated kinase (ERK), a subfamily of MAPKs, is thought to be mediating both E-LTP and L-LTP. It has been shown that many signaling cascades involved in E-LTP, including CaMKII and PKC, converge on ERK (Kelleher, Govindarajan, Jung, Kang, & Tonegawa, 2004). The need for a molecular coincidence explains the associative nature of LTP and the cellular mechanism for memory formation and consolidation.

1.2.2.3 Systems consolidation

While synaptic consolidation takes only minutes to hours, systems consolidation is a slower dynamic process that can take from weeks (in animals) to years (in humans). Systems

consolidation occurs at the systems level, suggesting the brain circuits gradually reorganizing (Frankland & Bontempi, 2005).

In humans, both declarative and non-declarative memories show time-dependent reorganization, although the timescales may be different (Scoville & Milner, 1957; Shadmehr & Holcomb, 1997). Similar time-dependent reorganization has been observed in animals as well. Memories such as courtship conditioning in flies and olfactory conditioning in bees have shown similar time-dependent reorganization (McBride et al., 1999; Menzel & Müller, 2001). As these examples indicate, systems consolidation may be a general phenomenon across species and different memory systems. However, in this dissertation, the discussion will be focused on the time-dependent reorganization within the hippocampal-dependent memory system in animal models, which much of the systems consolidation literature has been focused on.

1.2.2.3.1 Human studies for systems consolidation

Theodule Ribot suggested that memories might be gradually reorganized over time in 1882 (Ribot, 1882). He reported that in brain damage patients usually recently acquired memories were more readily lost than memories that were acquired in the more remote past. This pattern of temporally graded amnesia has become known as Ribot's law (or Ribot's gradient). In the well-known case of patient H.M., Penfield, Milner, and Scoville (1957) also described damage in the medial temporal lobe preferentially affects recent, but not remote, memories (Scoville & Milner, 1957). Importantly, H.M. showed complete amnesia for the memories within months before the surgery and more limited amnesia for the memories in the three years preceding the surgery (Scoville & Milner, 1957). Further analyses revealed that H.M.'s retrograde amnesia extended back approximately 11 years (Sagar et al., 1985), and his childhood memories are not affected. Along with H.M., comparable patterns of amnesia were reported in two other patients, who also received similar medial temporal lobe resection to treat psychotic diagnosis (Scoville & Milner, 1957). These observations suggested that damage in the medial temporal lobe often resulted in a temporally graded retrograde amnesia: it preferentially affected the recent memories (Kritchevsky & Squire, 1989).

Later studies used quantitative methods to characterize memory loss in patients with more restricted lesions and established that hippocampal damage, in particular, is typically associated with temporally graded retrograde amnesia (Beatty, Clouse, & Bierley, 1987; Rempel-Clower,

Zola, Squire, & Amaral, 1996; Salmon, Lasker, Butters, & Beatty, 1988; Squire, Slater, & Chace, 1975). Rempel-Clower et al. (1996) showed that the length of the temporal gradient might be mediated by the locus and extent of the brain damage, with large medial temporal lobe lesions resulted in the gradient covering decades (Rempel-Clower et al., 1996), but more restricted damage to the hippocampus typically associated with shorter gradients (Rempel-Clower et al. 1996). For example, in Kapur and Brooks' study (1999), two patients with restricted disease-induced hippocampal damage (assessed with magnetic resonance imaging) displayed retrograde amnesia limited to the few years preceding the brain damage (Kapur & Brooks, 1999). Manns et al. also showed that six patients with restricted lesions demonstrated impaired recent (several years preceding the onset of memory impairment), but intact remote (from 11–30 years before amnesia), memory when their recall of past news events was tested (Manns, Hopkins, & Squire, 2003). Same trend was also found in autobiographical memory (Bright et al., 2006; Kirwan, Wixted, & Squire, 2008). Collectively, these data suggest that the role of medial temporal lobe plays in memory is time-dependent.

Interestingly, the length of the gradient varies from several months to years, sometimes even decades. This is particularly critical as this various length may reflect the rate of reorganization and indicate how these memories reorganize. Several factors may affect the length of the gradient: First of all, it seems to be related to the extent of the damage (Squire & Alvarez, 1995). For instance, retrograde amnesia only extended back 1-2 years in two patients where the brain damage was only limited to the CA1 region of the hippocampus. On the contrary, patients with more extensive medial temporal lobe damage (including the entire hippocampus and parts of the entorhinal cortex) had a retrograde amnesia extending back to 15 years (Rempel-Clower et al., 1996). In some rare cases, the brain damage was beyond the medial temporal lobe. Accordingly, the retrograde amnesia was found flat, suggesting equal amnesia for recent and remote memories. In such cases, it is possible that the regions implicated in permanent memory storage are also affected (Squire & Alvarez, 1995). Secondly, the length of the gradient may also be related to the type of memory being tested (Bayley, Hopkins, & Squire, 2003; Nadel & Moscovitch, 1997; Rosenbaum et al., 2004). For instance, in the medial temporal lobe lesion patients, although the remote memories remained retrievable, they might not be as vivid when compared with healthy individuals (Viskontas, McAndrews, & Moscovitch, 2002).

1.2.2.3.2 Animal Studies for systems consolidation

Animal models have been key in dissociating the role of the medial temporal lobe in memory. In humans, damage to the medial temporal lobe has been shown to produce retrograde amnesia with a temporal gradient, at least for some forms of declarative memory. However, patient studies are limited in their capacity to further examine retrograde amnesia. First, because these studies rely on retrospective evaluations, it is difficult to compare performance across different time points. Second, the brain damage of patients varies from one case to another, and lesions are rarely confined to the hippocampus (Squire & Alvarez, 1995). To address these matters, animal models have been developed so that researchers can examine the relationship between hippocampal lesions and retrograde amnesia in a well-designed and well-controlled manner. As such, the experimenter can decide the location and extent of the lesions, the type and strength of the memory, and the timing of the learning phase and test phase. Generally, in the animal studies for systems consolidation, recent memory is defined as one to three days following learning, and remote memory is defined as 21-100 days following learning, depending on the species and the task. It is noteworthy that the timeframe for humans is very different from the animal studies.

Hippocampus and temporally-graded retrograde amnesia

Studies in primates, rats, mice, and rabbits have all demonstrated a time-limited role for the hippocampus in a range of spatial and non-spatial memory tasks (Squire & Bayley, 2007). In these studies, mainly socially-acquired food preference and contextual fear conditioning behavioral models have been used to assess memory (Frankland and Bontempi, 2005). Despite the differences between these tasks (i.e., properties of stimuli and performance), there are some common features with declarative memory in humans. In these tasks, animals are required recognize the relationships among the stimuli, forming memories that integrate the context (spatial and temporal information) (Ergorul & Eichenbaum, 2004).

The general pattern in these studies shows that impaired hippocampal functioning preferentially affects recent, but not remote, memories (Frankland & Bontempi, 2005). In one study done, Zola-Morgan and Squire (1990) trained monkeys to discriminate between five sets of object pairs at various intervals (between two and 16 weeks) prior to the removal of the hippocampal formation. As expected, the sham animals performed better for the recent associations, but poorer for the remotely acquired associations. Interestingly, in the hippocampal lesioned group,

memory for the recently acquired association was impaired and memory for the remotely acquired associations was spared (Zola-Morgan & Squire, 1990). Later that year, a study with rats investigated the time-limited role of the hippocampus using a socially acquired food preference paradigm. In this study, a naïve rat is paired with a demonstrator rat that has recently sampled a novel food. Following this interaction, the naïve rat tends to display a preference for that food. Lesions of the hippocampus performed either immediately after or three days following the food preference training impaired this memory. Interestingly, lesions performed five days following the interaction showed no effect (Winocur & Moscovitch, 1990).

Another behavioral model that has been used extensively to assess memory is contextual fear conditioning. In this paradigm, animals are conditioned to associate the environment (the conditioned stimulus, CS) with an aversive foot shock (the unconditioned stimulus, US). When the animal is returned to the CS, they display the specific freezing defensive response, defined as the complete lack of movement except for breathing. The time spent freezing during the test can be taken as an index of memory. One of the best-known studies to demonstrate temporally graded retrograde amnesia in animals was done by Kim and Fanselow in 1992, using a simple but elegant approach to examine the reorganization of contextual fear memory. In this study, rats received a hippocampal lesion 1, 7, 14, or 28 days following contextual fear conditioning. Rats that received lesions 1 day after conditioning (the recent group) displayed impaired contextual fear memory, whereas rats that received lesions 28 days following conditioning (the remote group) displayed freezing levels equivalent to the sham animals (Kim & Fanselow, 1992). This suggested that disrupting hippocampal function preferentially affects recent contextual fear memories. Similar results were observed in contextual fear conditioning using neurotoxic instead of the electrolytic lesions (Maren, Aharonov, & Fanselow, 1997), as well as in studies using a within-subject design (Anagnostaras, Maren, & Fanselow, 1999). Specifically, in the experiments by Anagnostaras et al., animals were trained in two highly distinctive contexts (previous work had indicated that there was little generalization between these two environments). Animals were first conditioned in context A, and then in context B seven weeks later. Excitotoxic hippocampal lesions were made shortly after the second contextual fear conditioning, and it was found that this lesion impaired only the recent memories (regardless of the order of context A and B). This within-subject design study further confirms that there is a temporal gradient in the retrograde amnesia in contextual fear conditioning (Anagnostaras et al.,

1999). Collectively, this abundant research has demonstrated that disrupting hippocampal function preferentially affects recent, but not remote, contextual fear memories (Frankland & Bontempi, 2005).

In fact, disruption of hippocampal function leads to a temporally graded retrograde amnesia in a variety of behavioral paradigms in animals, including trace fear conditioning (Kim, Clark, & Thompson, 1995), spatial discrimination (Cho, Beracochea, & Jaffard, 1993; Maviel, Durkin, Menzaghi, & Bontempi, 2004; Ramos, 1998;), visual discrimination (Wiig, Cooper, & Bear, 1996), trace eyeblink conditioning (Takehara, Kawahara, & Kirino, 2003; Takehara, Kawahara, Takatsuki, & Kirino, 2002), inhibitory avoidance (Quillfeldt et al., 1996), and in some variations of the water maze (Glenn, Nesbitt, & Mumby, 2003; Remondes & Schuman, 2004; Shimizu, Tang, Rampon, & Tsien, 2000; Yasuda & Mayford, 2006). These studies report a temporally graded retrograde amnesia consistent with the findings in patients with medial temporal lobe damage. The temporal gradient in retrograde amnesia has not only been shown in various behavioral paradigms, but also with various experimental approaches, including lesions, pharmacological interventions, and genetic approaches (Anagnostaras, Gale, & Fanselow, 2001; Debiec, LeDoux, & Nader, 2002; Maren et al., 1997; Quinn, Ma, Tinsley, Koch, & Fanselow, 2008; Wiltgen & Silva, 2007; Winocur, Moscovitch, & Sekeres, 2007). Extensive lesions in the hippocampus (Anagnostaras et al., 1999; Clark, Broadbent, Zola, & Squire, 2002), entorhinal cortex (Cho, Brown, & Bashir, 2002; Cho & Kesner, 1996), and perirhinal cortex (Glenn, Nesbitt, & Mumby, 2003; Thornton, Rothblat, & Murray, 1997) have also been investigated. In the animal studies, the length of the temporal gradient ranged from a few days to several weeks. Notably, this is much shorter compared to gradients observed in humans following damage to the medial temporal lobe. Again, the length of the gradient may depend on several factors, such as species, the nature of memory task, the intensity of training, and importantly the extent and location of lesion.

Cortex and temporally graded retrograde amnesia

Assuming there is a temporal gradient in memory and that the hippocampus is important for recent memories, then an obvious question might be, where are remote memories stored? If the old, remote memories are no longer dependent on the hippocampus, where are they? Is there a specific brain region critical for remote, but not recent, memory? To answer these questions, researchers adopted a parallel approach to those adopted in hippocampal studies.

Several studies have demonstrated that dysfunction of specific cortical regions disrupt remote, but not recent, memory (Bontempi, Laurent-Demir, Destrade, & Jaffard, 1999; Frankland, O'Brien, Ohno, Kirkwood, & Silva, 2001). The role of the hippocampus and medial prefrontal cortex in both recent and remote memory has been investigated using the trace eyeblink-conditioning paradigm. In the study done by Takehara et al. (2002), lesions in the hippocampus impaired the recent, but not the remote, trace-eyeblink conditioning. In contrast, lesions in the medial prefrontal cortex impaired the remote, but not recent, trace-eyeblink conditioning (Takehara et al., 2003). Using the contextual fear conditioning paradigm, Frankland et al. (2004) showed that inactivation of the anterior cingulate cortex disrupted remote (36 days old), but not recent (1 day old) contextual fear memory in mice (Frankland, Bontempi, Talton, & Kaczmarek, & Silva, 2004). A similar time-limited role for the anterior cingulate cortex was observed in the conditioned taste aversion paradigm with a parallel experimental design in mice (Ding, Teixeira, & Frankland, 2008). To investigate the role of the anterior cingulate cortex in remote memories, Restivo and colleagues (2009) examined the neuronal morphology following contextual fear conditioning. They demonstrated that recent memory was associated with increased dendritic spine formation in the hippocampus, while remote memory was associated with dendritic spine formation in the anterior cingulate cortex (Restivo, Vetere, Bontempi, & Ammassari-Teule, 2009). Furthermore, Vetere et al. (2011) showed that disrupting the spine growth in the anterior cingulate cortex after contextual fear conditioning blocked the subsequent memory expression (Vetere et al., 2011). These studies indicate that remote memory expression requires plasticity in the anterior cingulate cortex. In addition, it has been shown that an epigenetic mechanism in the anterior cingulate cortex is implicated in the maintenance of remote memory; infusion of DNA methyltransferase inhibitors into the anterior cingulate cortex prior to a remote memory test blocked memory expression (Miller et al., 2010). Other cortex regions have also been implicated in remote memory. For example, inactivation of the orbital frontal cortex impaired remote memory in the socially acquired food preference paradigm (Lesburguères et al., 2011). Consistent with the evidence above, lesions of auditory, visual, or olfactory secondary sensory cortices impaired remote, but not recent, emotion memory in mice (Sacco & Sacchetti, 2010). Collectively, these studies suggest that various cortical regions act as essential nodes in remote memory networks.

1.2.2.3.3 Ungraded retrograde amnesia

It should be noted that, in both human and animal model studies, a temporally graded retrograde amnesia is not always observed. It has been reported that some patients with medial temporal lobe damage display a flat retrograde amnesia, meaning that their memory of different time points was equally impaired. For example, in patient V.C., who had bilateral hippocampus damage, the assessment suggested that his memory for both general and personal facts and events was impaired, extending back four decades (Cipolotti et al., 2001). In another study, Noulhiane et al. (2007) examined autobiographical memory in a group of 22 patients who had undergone unilateral temporal lobe resection. Memory was reported to be impaired at all four time points covering the patients' lifespan (Noulhiane et al., 2007). These patients were likely to have more extensive brain damage that just the lateral temporal lobe and, as such, it is possible that regions responsible for permanent memory storage were also affected (Squire & Alvarez, 1995; Squire & Bayley, 2007).

In animal models, some studies also demonstrate flat gradient in hippocampus. For example, in spatial memory tasks, it was observed that hippocampal lesions impaired both recent and remote memory equivalently (Clark, Broadbent, & Squire, 2005; Martin, de Hoz, & Morris, 2005; Sutherland et al., 2001). A flat gradient in spatial and place navigation tasks in rodents has consistently been reported. Why is there no temporal gradient observed in spatial memory impairment? At least two explanations have been proposed. First, there is the possibility that the hippocampus is necessary for task performance, such as spatial navigation, in which the updating of current position is required during testing (Knowlton & Fanselow, 1998). For example, in more demanding spatial memory tests, the hippocampus might always be necessary online updating of position (or path integration) (Anagnostaras, Gale, & Fanselow, 2002). Second, it has been proposed that the permanent susceptibility of spatial memory to hippocampal disruption is because spatial memories are consolidated solely within the hippocampus and therefore always require hippocampal activity for their expression (Martin et al., 2005).

1.2.2.3.4 Models of systems consolidations

Several models and theories have been proposed to explain the gradual reorganization memory networks, a dynamic process that takes from weeks to years. David Marr proposed the first model to account for systems consolidation back in 1970. He thought that while the

hippocampus stores experiences during the day, the memories are played back to the cortex during sleep, allowing for the categorization subsequent reorganization of information. In his model, the process depended on the replay of waking patterns of neural activity during sleep (Marr,1970; Marr,1971). Within this theory, Marr proposed three essential concepts that become the foundation of memory models today: first, the hippocampus only stores information temporarily; second, the patterns of neural activity are replayed (the reinstatement or rehearsal of memory) while sleeping; third, the cortex is important in retrieving semantic facts of the memory (Frankland & Bontempi, 2005; McClelland, McNaughton, & O'Reilly, 1995; Squire & Alvarez, 1995).

To further elaborate on the interaction between the hippocampus and the cortex, Alvarez and Squire (1994) proposed that the hippocampus is necessary to co-activate the multiple cortical components involved in the representation of a previous event. After enough reactivations, these traces (which are distributed across the cortex) become bound together and can be expressed without further need of the hippocampus (Alvarez & Squire, 1994). Similar to this proposal, Murre (1996) proposed that the cortex consists of sparse modular connectivity, with a lack of direct connectivity between two cortical neurons. However, the communication between cortical neurons can be facilitated by indirect connections with the hippocampus (Murre, 1996).

In contrast, McClelland et al. (1995) suggested an alternative model for the interaction between the hippocampus and the cortex. They proposed that the information originally stored in the hippocampal system is gradually incorporated into the cortical system as a result of reactivation, and thus, cortical representation is not initially involved in memory consolidation. They further proposed that learning in the cortex must be slow and gradual to avoid any catastrophic interference. Catastrophic interference here refers to the phenomenon that occurs when later training disrupts the results of previous training, by rapidly integrating into a previously established network. The slow learning process of the cortex allows the integration of knowledge into a permanent cortical status (McClelland et al., 1995).

Below I elaborate on the dominant model of systems consolidation developed by Squire and Alvarez (“the standard model of systems consolidation”), as well as contemporary alternate views of systems consolidation.

Standard model of systems consolidation

The early and dominant view of systems consolidation was the standard model proposed by Squire and Alvarez (Squire & Alvarez, 1995). This standard theory of systems consolidation proposes that memories are reorganized in the brain over time, gradually becoming independent of the medial temporal lobe and instead supported by a distributed neocortical network. This model was developed from human neuropsychological studies and in animal models, and eventually stimulating the development of computational models to account for the process.

Evidence for this model comes from human studies in which patients with medial temporal lobe damage showed a temporally graded amnesia (Scoville & Milner, 1957; Zola-Morgan, Squire, & Amaral, 1986). This paradoxical finding of better memories for older information might suggest that the hippocampus is essential for reorganizing memory over time.

As the remote memory traces gradually transferred into cortex (McKee & Squire, 1992), the standard theory of systems consolidation hypothesizes that the hippocampus is required for strengthening the weak connections within cortex. The information is encoded in parallel and hippocampus serves as a potential index (Teyler & DiScenna, 1986). Interestingly, in recent neuropsychological patient studies, functional brain imaging data suggest hemodynamic activity in the hippocampus is highest for recent news events (3 years) but decreases with the age of the events (over a 30-year span) during the recall of semantic memories (Smith & Squire, 2009). Takashima et al. (2006) also showed that hippocampal activity for 90-day old memories is relatively lower compared to 1-day old information during memory recall. A similar pattern can also be found in the animal studies, refer to previous section where the evidence for the roles of the hippocampus and cortex in temporally graded retrograde amnesia is discussed.

In sum, according to the standard model of systems consolidation, memory is initially encoded both in the hippocampus and the cortex. Subsequent hippocampal network reinstates neuronal activity in the distributed cortical networks. This coordinated replay across the hippocampus and cortex leads to a gradual strengthening of the connections between cortical regions. A sufficient number of reactivations will eventually allow new memories to become more independent from the hippocampus and more dependent on the cortex. As a result, memories are assumed to decay more rapidly in the hippocampus than in the cortex, because hippocampal traces are not

strengthened by reactivation over time, but instead may become weaker through passive decay or interference (Frankland & Bontempi, 2005).

Multiple trace theory of systems consolidation

An alternative model of systems consolidation is the multiple trace theory (or the MTT model). This theory posits that, upon the instance of memory retrieval, a new neuronal trace would be formed by the hippocampus, no matter how remote the memory is. Nadel and Moscovitch (1997) advanced the multiple trace theory and stated that although the rough trace of a memory might be intact after hippocampal lesions, the detail and vividness of a memory requires the hippocampus (Nadel & Moscovitch, 1997).

The multiple trace theory is based on two observations. First, medial temporal lobe damage sometimes produces ungraded retrograde amnesia when declarative memory is being tested. Specifically, this has been reported in autobiographical and episodic (Cipolotti et al., 2001; Viskontas et al., 2002), as well as in detailed spatial memories (Martin et al., 2005; Rosenbaum et al., 2000). Second, imaging studies in humans indicate that the recall of remote, detailed autobiographical and episodic memories engages the hippocampus (Addis, McIntosh, Moscovitch, Crawley, & McAndrews, 2004; Gilboa, Winocur, Grady, Hevenor, & Moscovitch, 2004; Maguire & Frith, 2003; Ryan et al., 2001).

The multiple trace theory can account for these observations because it predicts that complete hippocampal lesions should produce temporally graded retrograde amnesia only in semantic, but not episodic and spatial, memories. The theory suggests that the hippocampus is always required for storage and retrieval of episodic and spatial memories, whereas semantic memory is mediated by the cortex alone and is dependent on the completion of the systems consolidation process after memory formation. This is supported by the study done by Moscovitch et al., where they examined the type of memory being probed in the studies reporting a temporally graded retrograde amnesia (Moscovitch, Nadel, Winocur, Gilboa, & Rosenbaum, 2006). This model suggests that different memory systems go through different processes of systems consolidation. While semantic memories undergo systems consolidation and become independent of the hippocampus over time, episodic and contextual memories always remain dependent on the hippocampus.

To account for the temporally graded retrograde amnesia for episodic memories, the multiple trace theory states that this would only be observed in cases where hippocampal damage is incomplete. It is suggested that each time the memory reactivates; a new trace is created by the hippocampus. Memory traces proliferate over time, and thus, when hippocampal damage is incomplete, older memories are less susceptible to disruption because they have more traces in the hippocampus (Nadel & Moscovitch, 1997).

However, one study on patient E.P. is inconsistent with the prediction of multiple trace theory. E.P. had extensive bilateral medial temporal lobe lesions, but had excellent autobiographical and spatial memories from his youth (Teng & Squire, 1999). However, there is still considerable debate whether the spared remote memories in patients like E.P. are as vivid and detailed as in healthy subjects (Rosenbaum et al., 2000).

Transformation hypothesis of systems consolidation

The transformation hypothesis can be seen as an updated version of the multiple trace theory. It proposes a modified framework to examine systems consolidation. The transformation hypothesis posits that the neuronal reactivation changes in systems consolidation are accompanied by changes in the memory itself (Winocur & Moscovitch, 2011; Winocur, Moscovitch, & Bontempi, 2010). This theory emphasizes a process of transformation: the initial detailed contextual memory is transformed into a less detailed memory over time, and these two traces can co-exist and interact. While the systems consolidation model states that when memory loses its hippocampal dependency over time, an identical version is formed in cortex (Squire & Alvarez, 1995), the transformation theory states that, some memories are transformed from the original ones that are episodic and context-specific to those that are semantic or schematic over time. During the process, the transformed memories are no longer hippocampal-dependent; instead, they are represented in other structures. However, the context-specific and episodic memories will continue to be dependent on the hippocampus. Thus, the retrieval of the semantic version of the memory does not require the hippocampus. As the transformation hypothesis proposes that both the episodic and semantic forms of memories may co-exist and interact dynamically with each other, we could have overall knowledge of an event while also retaining specific details paired with the original context of the event. Importantly, each form would be represented in its own respective neural system.

The transformation hypothesis proposes three critical elements different from the standard theory of systems consolidation: (1) the initial contextual memory trace remains dependent on the hippocampus for as long as it is available, (2) the hippocampal memory supports the development of the schematic version of the memory in the neocortex, and (3) there is a dynamic interplay between the two versions of memory, and one or the other may be more dominant, depending on the circumstances at retrieval.

Though the transformation hypothesis is built upon the multiple trace theory and is consistent with much of the evidence, some questions remain. For example, what is the role of the hippocampus in the transformation process? Winocur and Moscovitch (2007) proposed that the hippocampus might be important in retaining multiple representations of an episode. The general information abstracted may be stored in the neocortex and is thought to be the basis of semantic memory. Through repeated reactivation of these memories, either by retrieving them consciously or by replaying them during sleep, synaptic changes are strengthened and form the neural circuitry of semantic memories in the neocortex.

With this theory, some of the flat gradients observed in animal and human studies can be explained by the reliance of the memory on context and detail (Winocur et al., 2010). While episodic memory is difficult to definitively demonstrate in animals, a similarity can be drawn between episodic memory in humans and context-specific memory in rodents. Both forms of memory consist of representations that incorporate a detailed spatial-temporal context and which depend on the hippocampus (Rosenbaum, Winocur, & Moscovitch, 2001). Considering context-specific and context-general memories in animals to be the animal homologue of episodic and semantic memory in humans, respectively, the transformation hypothesis has directed a different approach to the study of systems consolidation.

According to the transformation hypothesis, the quality of memory depends on whether the hippocampus is involved in memory retrieval. It states that detailed, episodic memory will always require the hippocampus as long as the memory retains its specific context, such as where and when it was formed. Since the site and extent of brain damage varies across human amnesic patients, researchers have developed similar behavioral models in animal experiments. To test how the quality of memory changes over time, researchers have examined context-specificity,

with context specific and context-general memories in animals being the homologues of episodic and semantic memory in humans.

Contextual fear conditioning is one of the most studied paradigms, and it has been adapted to examine the quality, or the context specificity, of memory. To investigate the amount of detail retained, researchers have tested animals in different contexts: the original training context and other similar (but otherwise novel) contexts. How well the animals were able to discriminate between these contexts is then used as an index of how detailed or how specific their memory is. Wiltgen and Silva (2007) demonstrated that following contextual fear conditioning, the memory for the context generalized over time and lost its specificity. At the recent time point (1 day), animals showed more freezing in the training context compared to the novel context. However, when the animals were tested at increasing delays of up to five weeks following training, freezing levels in the novel context increased, resulting in equivalent freezing times in both contexts (Wiltgen & Silva, 2007). Similar findings have been reported by others (Biedenkapp & Rudy, 2007; Winocur et al., 2007), where contextual memory became less precise with time. Importantly, Wiltgen et al. (2010) further demonstrated that hippocampal inactivation impaired the expression of specific but not generalized memories in rats, thus indicating that the hippocampus plays a permanent role in the expression of detailed memories (Wiltgen et al., 2010). Once again using the contextual fear conditioning paradigm, Wiltgen et al. explored whether the original detailed memory still existed at the remote time points by placing the animals back into the conditioning context for one minute, as a reminder. With this reminder, the remote memory regained its specificity, implying that the original detailed contextual memory still remained intact and was accessible, at least following a reminder. In line with these results, Winocur et al. (2009) had showed that remote contextual memory returned to being hippocampus-dependent after a reminder via brief re-exposure to the original context, but not after exposure to another context (Winocur et al., 2009).

These results are consistent with the model proposed by McClelland et al. (1995), in which the reorganization over time of the brain regions supporting memory reflects the extraction of general properties from specific experiences (McClelland et al., 1995). These experiments employing the contextual fear conditioning paradigm along with reminders also support the transformation hypothesis. That is, memory can exist in two states: a hippocampus-dependent

detailed state and a hippocampal independent general state. Both states can co-exist simultaneously, with conditions determining which one dominates during memory expression.

The Schema assimilation model

Recent studies from Richard Morris's group have suggested that prior knowledge (the schema) might accelerate systems consolidation (Tse et al., 2007). This model considers the role of prior knowledge or the "mental schemas" in determining the speed with which systems consolidation takes place. The debate between the standard systems consolidation theory (Squire & Bayley, 2007) and multiple trace theory (Moscovitch et al., 2006) is ongoing and new findings have added to the discussion: first, whether the quality of memory changes over time and second, whether systems consolidation could occur on a shorter time scale. Toward this end, the first issue has been discussed in the previous sections; studies using the context fear conditioning paradigm suggest that recent memory tends to be more precise. The schema concept deals with the second issue and suggests that prior knowledge can accelerate the systems consolidation (Wang & Morris, 2010).

The schema concept emerged from work using the paired-associate learning paradigm in an "event arena." In these experiments, a schema of flavor-place associations was established with extensive training in the event arena. Once the schema was established, the expression of a single trial hippocampal dependent flavor-place association was disrupted by hippocampal lesions performed 3 hours after the single trial learning, but not when performed 48 hours after (Tse et al., 2007). This finding supports the idea of rapid systems consolidation. An alternative interpretation of these results is that the impaired association is due to an impaired synaptic consolidation. Based on the assumption that the cortex is sufficient to support the acquisition of the association in this task, it has been proposed that neurotoxic lesions to the hippocampus may cause disturbed neural activity outside the hippocampus, and disrupt the synaptic consolidation outside of the hippocampus (Rudy & Sutherland, 2008). However, it has been shown that the hippocampal lesioned animals were not able to perform this flavor-place association task, which indicates that the cortex alone is not sufficient to learn this task (Tse et al., 2008). It was found that this schema-dependent learning was associated with the up-regulation of immediate-early genes in the medial prefrontal cortex (Tse et al., 2011). More importantly, pharmacological intervention targeted at the medial prefrontal cortex prevented learning and the recall of consolidated memories.

Distributed Reinstatement Theory

The distributed reinstatement theory has also been proposed to account for how memories may become independent of the hippocampus. This theory emerges from the observation that the initial learning parameters, such as the distributed learning paradigm, may influence the susceptibility of the memory to hippocampal damage. For example, a recently published study by Lehmann et al. (2009) examining the effects of hippocampal damage on contextual fear conditioning in rats suggested that when training was distributed across several spaced sessions, the contextual fear memory was immune to hippocampal disruption at a recent delay. In contrast, animals that received training with equivalent context and shock exposure, but in a single session, were susceptible to retrograde amnesia induced by hippocampal lesions (Lehmann et al., 2009). In line with these results, a more recent study by the same researchers reported that fear memory was more resistant to hippocampal disruption after the reactivations (involving a brief return to the conditioning context without shock), suggesting that memory reactivations contribute to long-term memories becoming independent of the hippocampus (Lehmann & McNamara, 2011). These observations suggest that the distribution of the learning experience may influence the susceptibility of the memory to hippocampal damage (Sutherland et al., 2010). The distributed reinstatement theory states that each time the event is reinstated; the memory circuit is strengthened in non-hippocampal systems. Therefore, it is not the passage of time that is the central feature for how memories become independent of the hippocampus, but rather the number of reactivations. Although it is worth noting that the passage of time would generally allow for more opportunities for reactivation.

1.3 Conditioned place preference

1.3.1 Historical review of CPP

Conditioned place preference (CPP) has been developed to study the rewarding properties of drugs and has been widely used in addiction research (Tzschentke, 2007). In this behavioral paradigm, the animal forms an association between a neutral environment (conditioned context) and a positive/negative reward (in many cases, drugs) during training. In the test session, when given the choice of conditioned and non-conditioned contexts, animals prefer the drug-paired side and this preference can be quantified by time (Bardo & Bevins, 2000).

The first documented paradigm similar to place preference was designed by Thorndike (1911). He trained animals to go to a specific place in a box, unlock a latch, and then escape (Thorndike, 1911). However, current settings for place conditioning are quite different from this initial paradigm. One of the first experiments to use the current place preference settings to study how context becomes associated with drugs was done by Spragg (1940). In this study, two daily morphine injections were given to chimpanzees by the experimenter. As a result, the chimpanzees demonstrated drug-seeking behavior, such as moving toward the room and the syringe when exposed to the room (context) that was previously associated with morphine injection. Moreover, when presented with a choice of boxes associated with either a banana or with morphine, the chimpanzees recognized and chose the morphine-associated box (Spragg, 1940). However, there was no attempt made to formally quantify these behaviors. A study published in 1957 reported that rats could be trained to choose the morphine arm in a Y-maze task (Beach, 1957). In the same year, Garcia et al. (1957) reported that exposing rats to ionizing radiation in a distinctive environment induced an aversion to the cues of the environment (Garcia, Kimeldorf, & Hunt, 1957). The first study using a more "contemporary version" of CPP is from Rossi and Reid (1976), where they used the duration of time spent in the morphine-paired context relative to the saline-paired context as an index of morphine CPP (Rossi & Reid, 1976).

The CPP paradigm offers an alternative assessment of the rewarding properties of drugs and the number of studies using CPP as a behavioral model has increased from year to year. More than 1000 new studies using CPP have been published since 1998 (Tzschentke, 2007). Behavioral, pharmacological, and molecular studies have been conducted to further investigate the mechanisms underlying CPP.

It has been documented that a variety of drugs and natural stimuli can induce CPP or conditioned place aversion (CPA). For example, natural rewards such as food (Spyraki, Fibiger, & Phillips, 1982a), water (Ågmo, Federman, Navarro, Pudua, & Velazquez, 1993), social interaction (Calcagnetti & Schechter, 1992), wheel running (Antoniadis, Ko, Ralph, & McDonald, 2000), and sex (Meisel, Joppa, & Rowe, 1996) have been shown to induce CPP. Similarly, drug rewards, including cocaine (Mucha, van der Kooy, O'Shaughnessy, & Bucenieks, 1982; Nomikos & Spyraki, 1988), amphetamine (Spyraki, Fibiger, & Phillips 1982b), methamphetamine (Trazon, Suzuki, Misawa, & Watanabe, 1992), nicotine (Shoaib & Stolerman,

1994), ethanol (Black & Bonica, 1973; Howes & Reid, 1985), caffeine (Bedingfield et al., 1998), and morphine (Mucha et al., 1982) have induced CPP. On the other hand, aversive drugs induce conditioned place aversion, such as lithium chloride (Mucha et al., 1982), high doses of apomorphine (P. Best, M. Best, & Mickley, 1973), and naloxone (Mucha et al., 1982).

1.3.2 CPP procedures

The conditioned place preference paradigm has already been widely used to study the pharmacological properties of drug abuse. Although methodological details differ from laboratory to laboratory, typically the CPP procedure contains two essential phases: the conditioning phase and the test phase (Schechter & Calcagnetti, 1998). In the conditioning phase, the context (the conditioned stimulus, CS) becomes associated with the stimulus produced by the drug (the unconditioned stimulus, US) through a Pavlovian conditioning process (Eikelboom & Stewart, 1982; Koob, Sanna, & Bloom, 1998; Markou et al., 1993; Solomon and Corbit, 1974).

Conditioning Phase

During the conditioning phase, one of the two distinct contexts is paired with the stimuli of interest, such as cocaine in this study. Contextual cues, including visual cues, olfactory cues, and tactile cues (i.e., the texture of the floor, size or shape of the room, wall color or pattern) are used so that the animals are able to distinguish the two contexts. The conditioning itself involves an animal passively receiving the unconditioned stimulus (US; e.g., cocaine injection) repeatedly in one context (conditioned stimulus, CS), while the other context is presented without US. During the course of conditioning, the environmental cues become associated with the drug and act as the conditioned stimulus (CS).

Test Phase

During the test phase, the animals are presented with both contexts in the absence of the unconditioned stimuli. The animals will approach, avoid, or act neutrally towards the contexts. In general, pairing cues with appetitive rewards, such as drugs, results in an approach to those cues. However, place conditioning can also be used to detect the aversive properties of the stimuli. In this case, conditioned place aversion is observed (Tzschentke, 2007). Researchers can then measure the amount of time the animals spent in the conditioned context. Several indices have been used to report preference during the test sessions. The most common measures are: (1) raw time (i.e. time spent in each context), (2) percentage time spent, (3) difference between time

spent in the drug-paired and the vehicle-paired context, or (4) the difference between time spent in the pre-test and post-test in the drug-paired context. Cunningham et al. (2003) have discussed the rationales, advantages, and disadvantages for each of these measurements. They also provided an empirical comparison between using a biased or unbiased apparatus (Cunningham, Ferree, & Howard, 2003).

Biased and Unbiased Paradigms

It is important to note that animals may have a natural preference for one context even before conditioning begins. If this is the case, then the paradigm is said to be biased. In contrast, a paradigm is said to be unbiased should the conditioning stimuli (the context) be chosen in such a way as to produce approximately equal preferences for both contexts in naive (i.e., unconditioned) animals. Accordingly, it obviates the need for pre-testing these animals before conditioning since at baseline animals exhibit equal preference for either context (van der Kooy, 1987).

In the biased paradigm, a pre-test before conditioning is required. Due to the biased settings, there is often a substantial preference for one context over the other. In this case, the drug is paired with the least preferred context. Since counterbalancing is not possible, a separate control group (with vehicle injections) is often run to demonstrate that the shift of preference is drug-induced (Katz & Gormezano, 1979; Phillips & Le Paine, 1980, 1982; Wise & Bozarth, 1987).

In contrast, there are several advantages to using the unbiased paradigm. First, a pre-test is necessary for biased paradigm. According to latent inhibition theory, when presented with the context without the unconditioned stimuli, a context-US association forms. Thus non-rewarded pre-exposure to contexts may interfere with subsequent acquisition and expression of the context-drug associations (Bouton 1993; Lubow, Schnur, & Rifkin, 1976), resulting in a slowed acquisition of the CPP. Second, an animal's bias for a particular context might not be apparent on the initial test, but it may develop over time (Bardo & Bevins, 2000). Hence, using pre-test scores to assign which context is to be paired with which drug may be biased and inaccurate. With unbiased paradigm, such shift can be minimized by counterbalanced animals in experimental and control groups, and by comparing the drug- and vehicle-paired side instead of the pre- and post- conditioning. Specifcally, in the unbiased paradigm, the context paired with the drug can be counterbalanced in the group. That is, for each group of animals, half of them are

paired with context A, and the other half is paired with context B. As animals may develop a preference for any non-associative effects produced by drug exposure, for example chronic change in sensory-motor function or motivation, the counterbalanced experimental design provides a control for that (Cunningham et al., 2003). Because these groups are matched on exposure to the apparatus, the associated context, and the drug, but differ in the paired relation between each cue and drug, group differences are assumed to reflect the underlying context–drug association (Cunningham & Blomqvist, 2006).

Temporal Parameters

Similar to other forms of Pavlovian conditioning, several temporal parameters are important in CPP, including the time delay between the context exposure and drug effects (the so-called interstimulus interval (ISI) in Pavlovian conditioning), the duration of CS exposure, and the intertrial interval (ITI), i.e., is the time delay between conditioning sessions.

While these temporal parameters are important, they vary from laboratory to laboratory in the CPP protocol. First, in most CPP protocols, the ISI is short; the drug is delivered immediately or soon before exposure to the context in order to overlap the onset of the drug effects with the contextual cues. It has been shown that no conditioning is produced if the drug was delivered at a long delay before or after exposure to context (Bormann & Cunningham, 1997; Bardo & Bevins, 2000). Second, place preference has been demonstrated over a wide range of context exposure duration. In most protocols, it is between 10 and 30 min and depends on the drug of interest and the subject used. Third, the ITI in CPP typically ranges from hours to days. In this way, it is different from the Pavlovian fear conditioning, where the ITIs are often seconds or minutes. In CPP, usually there is a 24-hour interval between each conditioning session. This provides adequate time for the drug effects to be reset and allows the drug and the vehicle to be delivered at the same time of day to avoid introducing any circadian variables (Tzschentke, 2007; Cunningham, Groblewski, & Voorhees, 2011).

1.3.3 Interpretation of CPP

1.3.3.1 Novelty-seeking

A major concern of CPP is the potential influence of novelty-seeking behavior on the test day. It has been demonstrated that rodents, in general, prefer a novel context to a familiar context

(Bowling, Rowlett, & Bardo, 1993; Hughes, 1968; Parker, 1992). Pairing the drug with one context potentially interferes with the familiarization to that context, therefore rendering it a more novel context compared to the saline context on the drug-free test day. To address this issue, animals can be tested in an apparatus that has three distinct contexts: novel, drug paired, and saline paired. Importantly, when tested with this apparatus, animals show a preference for the drug-paired context over the novel context (Mucha & Iversen, 1984; Parker, 1992). Moreover, drugs like morphine and naloxone produce CPA, when tested either with or without the drug, suggesting that the novelty effect may not be robust enough to confound the place conditioning behavior (Mucha et al., 1982). It has also been reported that rats do not show a preference for a novel environment over one in which they had previously spent 30 min daily for 4 consecutive days (Mucha et al., 1982). Collectively, these data suggest that novelty seeking does not confound the interpretation of the CPP paradigm.

1.3.3.2 State-dependent learning

Another concern about CPP is from the perspective of state-dependent learning. That is, the drug effect plays an essential role in determining the novelty (Berlyne, 1969). This theory posits that in the conditioning process, when under the influence of the drug (CXT+), the context paired with the drug is less novel, and the context paired with the vehicle (CXT-) is relatively novel. When not under the influence of the drug, however, the context paired with the drug is now more novel and the context paired with vehicle is less novel. Therefore, one would predict that when tested drug-free, the animal prefers the CXT+ because of novelty. However, the state-dependent learning theory predicts that when tested under the influence of the drug, animals would prefer the CXT-. Importantly, it has been shown that animals prefer the drug-paired context when tested, regardless of whether hey are tested with or without the drug (Mucha et al., 1982; Mucha & Iversen, 1984).

1.3.3.3 Conditioned inhibitor

It is widely accepted that, in CPP, the CS becomes associated with the drug through a Pavlovian conditioning process (Eikelboom & Stewart, 1982; Solomon & Corbit, 1974). However, it is also possible that during the conditioning, the CXT- may act as a conditioned inhibitor (Stewart, 1992; Wagner, Mazur, Donegan, & Pfautz, 1980).

The conditioned inhibitor occurs when the stimulus is presented in the presence of CS and is not followed by the US (Rescorla, 1969). In the case of CPP, the CXT- is clearly not followed by the US. But whether the CXT- is presented with some components of CS is somewhat less obvious. For example, the transportation, handling, injection, and the experimenter could each be a part of the CS and these tend to be the same in both CXT- and CXT+. It is possible that these factors acquire an excitatory association with the US. In this case, CXT- is presented in the presence of CS, but not followed by the US, and thus may become a conditioned inhibitor during the test phase of CPP. However, if the conditioned inhibitor CXT- were the sole reason for the place preference, then one would predict equal or more preference for the novel context compared to the drug-paired context when presented with any of the three contexts (CXT+, CXT-, novel). It has been shown, however, that even when presented which each of the contexts, there is a preference for the CXT+ to the novel context (Mucha & Iversen, 1984; Parker, 1992). Therefore, the conditioned inhibitor is not the sole reason of the CPP.

1.3.4 Hippocampus and nucleus accumbens in CPP

1.3.4.1 Hippocampus and CPP

The hippocampus has been recognized as a central structure in conditioning and memory, but it is not typically associated with drug addiction. However, accumulating evidence in lesion, inactivation, immediate early gene (IEG), and electrophysiological studies has shown that the hippocampus plays a role in CPP. This is not unexpected, since CPP depends on context-drug associations, and the hippocampus has been suggested to play a critical role in context conditioning. In fact, contextual information processing and reward-related memory formation and retrieval are essential in CPP, and these functions may be hippocampus-dependent (Jones & Wilson, 2005; Lansink, Goltstein, Lankelma, McNaughton, & Pennartz, 2009; Masuoka, Fujii, & Kamei, 2006). The hippocampus has been proposed to send contextual information associated with drug effects to the nucleus accumbens (Everitt & Robbins, 2005), a part of the mesolimbic dopaminergic system that contributes crucially to CPP (Spyraki et al., 1982b). This is further supported by the evidence showing that the hippocampus is anatomically and functionally connected to the midbrain dopamine system. The hippocampus receives dopaminergic projection from the ventral tegmental area (VTA) (Baulac, Verney, & Berger, 1986; Gasbarri, Verney, Innocenzi, Campana, & Pacitti, 1994; Gasbarri, Sulli, Innocenzi, Pacitti, & Brioni, 1996) and the nucleus accumbens receives glutamatergic afferents from the subiculum of the hippocampal

formation (Groenewegen, Vermeulen-Van der Zee, te Kortschot, & Witter, 1987). It also has been further demonstrated that brief electrical stimulation in the subiculum can evoke a significant increase in dopamine efflux in the nucleus accumbens, further suggesting that the hippocampus plays a part in drug CPP (Blaha, Yang, Floresco, Barr, & Phillips, 1997; Taepavarapruk, Floresco, & Phillips, 2000).

It has be shown that the dorsal and ventral hippocampus may differ functionally and anatomically (Bannerman et al., 1999; M. Moser and E. Moser, 1998). Lesions of the ventral, but not dorsal, hippocampus increase dopamine neurotransmission in the nucleus accumbens (Lipska et al., 1991; Lipska, Jaskiw, Chrapusta, Karoum, & Weinberger, 1992), which may in turn affect cocaine reward (Wise, 1984) and acquisition of cocaine CPP. Lesions to the hippocampus performed before training disrupt cocaine CPP acquisition (Hernández-Rabaza et al., 2008; Meyers, Zavala, & Neisewander, 2003), consistent with the disruptive effects of pre-training hippocampal lesions in food CPP (Ferbinteanu & McDonald, 2001) and amphetamine CPP (Le Pen, Gaudet, Mortas, Mory, & Moreau, 2002). Interestingly, the colchicine-induced lesions, when restricted to the dorsal dentate gyrus abolished cocaine (20 mg/kg i.p.) induced CPP acquisition and contextual fear conditioning in rats (Hernández-Rabaza et al., 2008). This is consistent with the report that NMDA lesions of the dorsal, but not ventral, hippocampus reduced cocaine (15 mg/kg i.p.) CPP in rats (Meyers, Zavala, & Neisewander, 2003).
In pharmacological intervention studies, it has be shown that temporary inactivation of the dorsal hippocampus with the γ -aminobutyric acid agonist muscimol blocked the acquisition and expression of cocaine-induced CPP when applied pre-training and pre-test, but not when applied post-training (Meyers, Zavala, Speer, & Neisewander, 2006). Studies have also shown that microinjections of dopamine receptor 1 antagonist into the hippocampus impaired morphine and methamphetamine induced CPP (Rezayof, Zarrindast, Sahraei, & Haeri-Rohani, 2003; Ricoy & Martinez, 2009). In addition, the protein kinase A and CREB are reported to increase in the hippocampus in cocaine CPP (Tropea, Kosofsky, & Rajadhyaksha, 2008). An indirect, but related, line of evidence supporting the role of hippocampus in drug-related memory comes from the context-primed reinstatement studies using the self-administration model. Here it has been shown that inactivation of the hippocampus impairs the expression, reconsolidation, and reinstatement of context-induced cocaine-seeking behavior (Fuchs et al., 2005; Fuchs, Eaddy,

Su, & Bell, 2007; Lasseter, Xie, Ramirez, & Fuchs, 2010; Ramirez et al., 2009), suggesting that the hippocampus may play an essential role in cocaine-context associations.

Studies examining expression of activity-regulated genes have also suggested that the hippocampus is important in the cocaine-context association. For example, FOS protein expression is increased in CA1, dentate gyrus, and dorsal and ventral subiculum in animals exposed to cocaine-associated environmental stimuli (Franklin & Druhan, 2000; Neisewander et al., 2000). Zif268 and the activity-regulated cytoskeleton-associated gene (arc) are transiently induced by cocaine-associated stimuli (Everitt & Robbins, 2000; Hearing, See, & Mcginty 2008; Zavala et al., 2008) and have been implicated in mediating cellular events that underlie the drug-associated learning processes (Lee, 2006; Lee, Ciano, & Thomas, 2005; Hearing et al., 2008; Zavala, Browning, Dickey, Biswas, & Neisewander, 2008). Moreover, human imaging studies in cocaine addicts have shown that the craving induced by cocaine-paired cues is accompanied by increased blood flow or metabolic activity in the hippocampus (Kilts et al., 2001).

Electrophysiological studies have also demonstrated that the oscillatory electroencephalography in the hippocampus is associated with the acquisition and expression of cocaine-induced CPP in the intact rat (Takano, Tanaka, Takano, & Hironaka, 2010). In addition, Vorel et al. (2001) reported that the stimulation of the hippocampus at the theta frequency caused a relapse to cocaine-seeking in the drug-taking context, suggesting that the hippocampus might encode an association between context and drug-induced hedonic states (Vorel, Liu, Hayes, Spector, & Gardner, 2001).

Hippocampus and contextual discrimination

Conditioned place preference could also be viewed as an appetitive contextual conditioning model, or to be more specific, a contextual discrimination model. Converging evidence suggests that the hippocampus may play an important role in a variety of forms of contextual conditioning (Anagnostaras et al., 1999; Kim & Fanselow, 1992; Maren et al., 1997; Otto & Poon, 2006; Parsons & Otto, 2008; Phillips & LeDoux, 1992; Rempel-Clower et al., 1996; Wang, Teixeira, Wheeler& Frankland, 2009; Winocur et al., 2009; Winocur & Moscovitch, 1990; Winocur et al., 2007; Zola-Morgan & Squire, 1990). Indeed, the hippocampus has been proposed to be essential for a polymodal representation of context (Rudy & Sutherland, 1989), which can then be associated with the US (Maren & Fanselow, 1995; Rogan & LeDoux, 1996). That is, the

hippocampus is required for the formation of a unified representation of the visual, olfactory, tactile, and auditory cues present in the training environment. Some studies indicate that pre-training electrolytic or excitotoxic lesions of the dorsal hippocampus profoundly impair the acquisition of contextual fear conditioning. These data are mostly compatible with the effects of hippocampal lesions on contextual conditioning in appetitive-motivated tasks (Good & Bannerman, 1997; Honey & Good, 1993).

However, these anterograde effects are not universally observed (Anagnostaras et al., 2001; Bast, Zhang, & Feldon, 2003; Cho, Friedman, & Silva, 1999; Frankland, Cestari, Filipkowski, McDonald, & Silva, 1998; Kim, Rison, & Fanselow, 1993; Maren et al., 1997; Maren, Anagnostaras, & Fanselow 1998; Phillips & LeDoux, 1992; Young, Bohenek, & Fanselow, 1994). In several studies, pre-training hippocampal manipulations impair the acquisition of contextual conditioning (Kim et al., 1993; Otto & Poon, 2006; Parsons & Otto, 2008; Phillips & LeDoux, 1992), while other studies show that such manipulations result in sparing, or only mild impairment, of contextual conditioning (Anagnostaras et al., 2001; Cho et al., 1999; Frankland et al., 1998; Maren et al., 1997; Wiltgen, Sanders, Anagnostaras, Sage, & Fanselow, 2006).

It has been suggested that these discrepancies may result from the discriminative nature of the task and may reflect the role of the hippocampus in discriminative contextual conditioning (Antoniadis & McDonald, 2000; Frankland et al., 1998; McDonald, King, Wasiak, Zelinski, & Hong, 2007; Otto & Poon, 2006; Parsons & Otto, 2008; Wang et al., 2009). That is, the contextual conditioning in animals with hippocampal damage may be processed by the conditioning to a single, unimodal cue embedded within that context (Maren et al., 1997).

In context discrimination, animals are trained to discriminate between two similar chambers, one in which they are shocked and the other in which they are not. An important feature of this task is that the two chambers consist of both unique and shared contextual cues so that the animals have to distinguish the context that predicts shock. Frankland et al. reported that pre-training hippocampal lesions impaired the acquisition of discriminative contextual fear conditioning but not the acquisition of non-discriminative contextual fear conditioning (Frankland et al., 1998). These data suggest that contextual fear conditioning is more likely to depend on hippocampal integrity when discrimination between multiple contextual stimuli is required. On the contrary, if a cue-based elemental solution is possible and context discrimination is not required, contextual

fear conditioning may not be impaired by hippocampal lesions (Anagnostaras et al., 2000; Frankland et al., 1998; Maren et al., 1997; Maren et al., 1998).

The temporal role of the hippocampus in contextual discrimination

Another feature of the hippocampus in contextual conditioning is its temporal involvement. It has been suggested that the hippocampus is initially essential for the expression of a recently acquired contextual conditioning memory but not for recall at later, more remote time points. Post-training manipulations of the hippocampus often, but not always, result in a more severe impairment in recently acquired contextual conditioning memory (Anagnostaras et al., 1999; Kim & Fanselow, 1992; Maren et al., 1997). This general pattern of evidence is consistent with the idea in systems consolidations that the hippocampus plays a time-specific role in memory, which has been demonstrated in both rodents and primates. However, other data suggest that memory is equally affected by the hippocampus at recent and remote times (Sutherland et al., 2008; Sutherland et al., 2010). These conflicting data may be due to inconsistency both within and between laboratories, such as the extent of the hippocampal damage, the delay between training, and the type of memory model used. It is also possible that the hippocampus plays different roles in the discriminative and non-discriminative contextual conditioning paradigms (Parsons & Otto, 2010). Importantly, hippocampal participation in the maintenance of discriminative contextual conditioning appears to be temporally graded (Parsons & Otto, 2010; Wang et al., 2009).

1.3.4.2 Nucleus accumbens

It has been shown that the mesolimbic dopaminergic system plays as a fundamental role in reward and addiction (Baik 2013; Pierce & Kumaresan, 2006; Wise 2004). The dopaminergic neurons from the VTA projecting to the nucleus accumbens have been indicated as the mesolimbic circuitry. The mesolimbic circuit connects the nucleus accumbens, medial prefrontal cortex, and hippocampus (Pierce & Kumaresan, 2006). It has been shown that cocaine elevates dopamine levels in the nucleus accumbens and thus activates these reward circuits. Moreover, the nucleus accumbens is also critical for the locomotor stimulant effects of cocaine (Everitt & Wolf, 2002; Koob et al., 1998; Wise, 2004). Specifically, NAc plays a critical role in mediating physiological effects of cocaine and cocaine-induced addiction behavior such as locomotor

sensitization, self-administration, and conditioned place preference (Wise, 2004; Pierce and Kumaresan, 2006).

Lesion studies have shown that pre-training lesions in the nucleus accumbens shell impair the acquisition and expression of amphetamine-induced CPP, suggesting that the nucleus accumbens might play an important role in the acquisition and expression of CPP induced by psychostimulants (Sellings and Clarke, 2003). Further work from the same group reported similar effects of pre-training lesions on cocaine CPP acquisition and locomotor activation (Sellings, 2006). Evidence from pharmacological manipulation studies shows that pre-training and pre-test inactivation of the NAc with the glutamate antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) impairs the acquisition and expression of cocaine CPP (Kaddis et al., 1995). Along with many others research, these data suggest that both glutamatergic and dopaminergic pathways in the NAc are essential in cocaine CPP. Moreover, another study by Hikida et al. (2001) showed that ablation of the cholinergic interneurons in the nucleus accumbens by immunotoxin-mediated cell targeting enhanced the cocaine (5 and 10 mg/kg, i.p.) CPP in mice (Hikida et al., 2001), suggesting that nucleus accumbens is important in different stages of the development of cocaine CPP and various neurotransmission pathways might be involved.

1.4 Systems consolidation of drug-associated contextual memories

1.4.1 Drug-associated contextual memories

Memory is thought to be an important component of drug addiction and relapse. Relapse is a critical obstacle in the long-term treatment for drug addiction (Dackis & O'Brien, 2001). It has been shown that the likelihood of returning to substance abuse remains high even after months or years of abstinence. Many studies using human and animal models have shown that exposure to contextual stimuli which have been associated with previous drug use, drug effects, or drug withdrawal symptoms can provoke strong drug seeking behavior or relapse to the drug in the abstinent abuser (Childress et al., 1993; Childress et al., 1999; Stewart, de Wit, & Eikelboom, 1984; Wikler, 1973). While the majority of addiction studies focus on reward, the memory component of addiction is less understood. It has been proposed that memory may play a part in the preoccupation and anticipation phase of addiction. Drugs of abuse may also induce

neuroadaptations in the learning and memory circuits, as shown in behavioral, cellular, and molecular studies (Hyman, 2005; Hyman, Malenka, & Nestler, 2006; Kalivas & McFarland, 2003; Koob & Volkow, 2010; Robbins, Ersche, & Everitt, 2008).

Studies in humans have suggested that exposure to the context originally associated with drug use can bring the memories of drug effects back and further induce strong craving and drug-seeking behaviors (Childress et al., 1993). These drug-associated CS, such as environmental stimuli and contexts that have been paired previously with drug use, can activate limbic corticostriatal circuitry in abstinent human addicts (Childress et al., 1999). For example, recent studies have shown that when exposed to the drug-associated context, the reactivation of the aversive memory of withdrawal may motivate drug-seeking behavior (Hellemans, Everitt, & Lee, 2006). Moreover, these memories for the drug-associated cues can be highly resistant to extinction and may be capable of inducing conditioned physiological responses (Ehrman et al., 1992; O'Brien, Childress, Ehrman, & Robbins, 1998).

Consistent with these studies, drug-associated CSs induce relapse behavior in many different animal models of addiction (Crombag & Shaham, 2002; Stewart et al., 1984), including cue-induced CPP (Lu et al., 2005) and second-order schedules self-administration (Everitt & Robbins, 2000; Markou et al., 1993). In addition, drug-associated CSs also activate the same key corticostriatal regions in animal models (Boujabit, Bontempi, Destrade, & Gisquet-Verrier, 2003). In the ABA renewal model (Bouton & Bolles, 1979; Pickens et al., 2011), rodents are first trained in context A, so that those contextual cues will be associated with the drug of interest. After training, the rodents go through the extinction phase in a different context B, which contains distinct tactile, visual, auditory, olfactory, or circadian features. After extinction, rodents are presented with the original drug-associated context A and drug-seeking behavior is assessed. Note that in the training phase, the drug is sometimes associated with an explicit, discrete drug cue (a light cue or a compound tone–light cue). Researchers have termed these two procedures the “cue-induced” or the “context-induced” reinstatement. Though it has been suggested that these two procedures may have different mechanisms (Crombag, Bossert, Koya, & Shaham, 2008), these procedures all show the same drug-associated contextual memories in addiction.

With different variations of the ABA procedure, researchers have shown that drug-associated contextual memories can induce reinstatement of different drugs: cocaine (Crombag, Grimm, & Shaham, 2002; Fletcher, Rizos, Sinyard, Tampakeras, & Higgins, 2008; Fuchs et al., 2005, 2007; Hamlin, Clemens, & Mcnally, 2008; Kearns & Weiss, 2007), heroin (Bossert, Ghitza, Lu, Epstein, & Shaham, 2005; Bossert, Poles, Wihbey, Koya, & Shaham, 2007), speedball (Crombag et al., 2002), alcohol (Zironi, Burattini, Aicardi, & Janak, 2006), and nicotine (Diergaarde, W. De Vries, Raasø, & T. De Vries, 2008). This ABA procedure has been reliably replicated across different contextual manipulations, different training and test conditions, and different drug classes.

The importance of drug-associated contextual memories in reinstatement has also been shown from IEG studies. Evidence suggests that exposure to drugs of abuse may lead to short-term and long-term adaptations of gene expression in the memory circuits. For example, cocaine-associated stimuli can induce the transient activation of activity-regulated IEGs, such as zif268 and arc, in the brain regions responsible for memory (Everitt & Robbins, 2000; Hearing, Schochet, See, & McGinty, 2010; Hearing, See, & Mcginty, 2008; Zavala et al., 2008). Studies also suggest that the activation of the IEGs may also mediate the cellular events involved in the process of drug and contextual cues association and events further involved in the drug-seeking behavior (Berke & Hyman, 2000; Hearing et al., 2008; Lee, 2006; Lee, Di Ciano, Thomas, & Everitt, 2005; Zavala et al., 2008).

The conditioning of context and drugs

The context-conditioned drug response has been studied across different classes of drugs. Aside contextual cues associated with drug experience eliciting drug cravings (Grüsser, Heinz, & Flor, 2000; Negrete & Emil, 1992) and inducing relapse in both humans (Childress et al., 1993; Hyman, 2005) and animals (Meil & See, 1996; Tran-Nguyen et al., 1998; Weiss et al., 2000), there are other drug-related physiological and behavioral effects evoked in a context-specific manner. For example, the context can induce body temperature or pain sensitivity changes in opiate addiction cases (Bespalov, Zvartau, & Beardsley, 2001). Behaviorally, locomotor activity and operant behavior changes have also be observed when exposed to a specific context previously associated with drugs (Pickens & Crowder, 1967; Siegel, 1975). Understanding the contextual memories associated with the drug, and how these memories develop over time is crucial, though still poorly understood.

1.4.2 The systems consolidation of drug-associated memory

There are several lines of evidence suggesting that drug-associated memories may change over time, like other types of memories. For example, studies in both human and animal models show that cravings for drugs develop over time. These cravings increase during abstinence and could evoke the drug-seeking behavior (Grimm, Hope, Wise, & Shaham, 2001; Shalev, Morales, Hope, Yap, & Shaham, 2001). Drug-seeking behavior after a long period of abstinence has been implicated in the transition from casual drug use to compulsive behaviors (Grimm et al., 2001; Thomas, Kalivas, & Shaham, 2008; Tran-Nguyen et al., 1998). Furthermore, repeated reflection on drug-related memories predicts future increases in substance abuse symptoms (Nolen-Hoeksema, Stice, Wade, & Bohon, 2007). This increase in craving has been reported not only for cocaine, but also for several other drugs, including methamphetamine (Shepard, Bossert, Liu, & Shaham, 2004), alcohol (Bienkowski et al., 2004), nicotine (Abdolahi, Acosta, Breslin, Hemby, & Lynch, 2010), and sucrose self-administration (Grimm, Fyall, & Osincup, 2005). These behavioral studies suggest that the circuits and the molecular mechanisms underlying drug-associated contextual memories may change over time.

Consistent with this, immediate early gene studies suggest that the circuits supporting drug-associated memories change in a time-dependent manner. Hearing et al. (2008) first reported that re-exposure to the environment previously associated with cocaine self-administration after 22 h or 15 days of abstinence produced a significant increase in zif268 and arc in the caudate-putamen and nucleus accumbens. Importantly, the expression pattern was differentially affected at both time points, suggesting changes in organization of this drug-associated memory (Hearing et al., 2008). The same group later reported similar observations in the hippocampus. This time, rats were exposed to an alternative context in the abstinence period. On post-conditioning days 8-14, all rats were transported to an alternate environment that was distinctly different from the self-administration room and were placed into clear plastic holding chambers for 2 hours per day, with no stimuli or cues present. On post-conditioning day 15, the rats were returned to the alternate environment, the self-administration chamber with no levers available, or the self-administration chamber with the levers available for 1 h. Hearing et al. (2010) reported that arc mRNA and zif268 activity was increased in the hippocampus after both twenty-two hours and fifteen days of cocaine abstinence (Hearing et al., 2010). Given the exposure to the alternative

context during abstinence, this result indicates that the hippocampus is involved in the context-induced reinstatement in self-administration at least up to 15 days following training.

In a recent study, Lucas et al. (2012) compared the neural circuits that are reactivated by the re-exposure of previously morphine-dependent rats to the withdrawal-paired environment 1 month after conditioning with 1 day after conditioning data (Frenois, Le Moine, & Cador, 2005; Lucas, Frenois, Cador, & Le Moine, 2012). They found that the neuronal circuits involved in the retrieval of the 30-day memories were somewhat different with the 1-day reactivation, with a shift from extended amygdala regions toward cortical areas. These data suggesting that memories linked to opiate withdrawal may change over time. To date, there is no systematic study examining the cocaine CPP memory reorganization or systems consolidation.

1.4.3 CPP as a model to study drug-associated contextual conditioning

1.4.3.1 Conditioned place preference as an analogous model of contextual fear conditioning

Mounting evidence suggests that the hippocampus plays an important role in contextual fear conditioning (Anagnostaras et al., 1999; Frankland et al. 1998; Kim & Fanselow, 1992; Phillips & LeDoux, 1992; Maren, et al., 1997; Rempel-Clower, et al., 1996; Wang, et al., 2009; Winocur, et al., 2009; Winocur, et al., 2007; Zola-Morgan & Squire, 1990**)**. While different protocols of contextual fear conditioning have been tested, the role of the hippocampus in context conditioning pairing with an appetitive stimulus has not been extensively addressed. Recently, the discriminative version of fear conditioning has been used to investigate systems consolidation and memory reorganization, as this protocol gives an indication about how well animals remember context (Winocur et al., 2009; Wang et al., 2009).

Conditioned place preference is a discriminative contextual conditioning process by nature, as it requires the animal to distinguish between two contexts. Conditioned place preference also shares common features with discriminative contextual fear conditioning, including that the environmental cues serve as CS and are associated with the US (e.g., drug effects). Operationally, in CPP, the context is the behavioral chambers that are used as the CS on the test day. Conceptually, the drug-paired context serves as a set of stimuli that are static and in the

background during training with no explicit, discrete signals used. Moreover, since the drug is administered by the experimenter before presentation of the context, the contextual stimuli are more temporally and spatially diffuse and are relatively less predictive than in self-administration. Experimentally, most CPP apparatuses use compound CSs composed of several elements from different sensory modalities including visual, olfactory, and tactile cues (Cunningham et al., 2011). Based on these similarities, CPP is thought to be analogous to the contextual discriminative fear conditioning paradigms.

Conditioned place preference for drugs of abuse can be used to study systems consolidation of drug-associated contextual conditioning, because it appears to be hippocampus-dependent at the initial stage of formation (Meyers et al., 2003; Meyers et al., 2006). First, the hippocampus exhibits neuronal activation associated with cocaine seeking behavior when in a previous drug-paired environment (Neisewander et al., 2000). Moreover, IEG studies also showed that exposure to drugs activates both short-term and long-term regulation of gene expression in the hippocampus; zif268 and arc are transiently induced by cocaine-associated stimuli (Everitt & Robbins, 2000; Hearing et al., 2008; Zavala et al., 2008). Second, pre-training lesions of the hippocampus impairs place conditioning (Cohen & Squire, 1980; Hernández-Rabaza et al., 2008; Meyers et al., 2003). Third, besides CPP, Fuchs et al. (2005) also reported that application of the sodium channel blocker tetrodotoxin in the dorsal hippocampus attenuated context-induced drug reinstatement in self-administration (Fuchs et al., 2005). However, this evidence is indirect and definitive evidence for CPP being dependent on the hippocampus and its exact role in the expression of recent and remote drug-associated contextual memories is lacking.

1.4.3.2 Comparison to the self-administration reinstatement paradigms

The self-administration paradigm has been widely used to study drug-associated contextual memory in rodents. Compared to the self-administration paradigm, CPP offers both advantages and disadvantages to approaching how the drug-associated contextual memories are formed and developed over time.

Compared to the self-administration, CPP measures the rewarding properties of the drug of interest by indexing drug-elicited approach and contact responses toward the associated

rewarding stimuli. It is based on the principles of classical conditioning. The self-administration model, on the other hand, reflects the reinforcing properties of the drug and is based on the operant principles of learning. While the self-administration procedures provide a direct way to assess the reinforcing features of the drug, CPP essentially assesses the association between the drug and the contextual stimuli. Another feature of CPP is that the drug is administered by the experimenter, whereas in the self-administration models, drug intake is a result of an operant response from the animal. Because the drug is injected passively by the experimenter in CPP (and thus, the animals do not have control over the intake of the drug), this model does not necessarily reflect human addiction behavior. However, it has been shown that experimenter-administered drugs are sufficient to produce many factors of addiction, including increasing motivation for the drug (Vezina, 2004), producing incentive sensitization to the cue (Di Ciano, 2008; Robinson & Berridge, 2000), impairing cognitive functions (Schoenbaum & Shaham, 2008), and resulting in stronger stimuli-response habits (Miles, Everitt, & Dickinson, 2003; Nelson & Killcross, 2006). Moreover, these studies have shown that experimenter-administered drug injections could induce sensitization and further induce neuroadaptations in the reward brain circuits in response to relapse (Paulson & Robinson, 1995; Pierce, Bell, Duffy, & Kalivas, 1996). Most importantly, the amount of the drug and the time of drug intake are controlled by the experimenter in CPP.

There are some additional advantages to using CPP to study drug-associated contextual memory and its role in relapse. First, the amount of drug intake and the drug effects are well controlled among animals and groups. Second, given that the current research focus is to understand the contextual associations with drugs, the fact that CPP is based on Pavlovian conditioning provides a model to directly examine the association. Third, there are many similarities between contextual fear conditioning and CPP. The processes mediating the associations of the context with a positive stimulus (such as cocaine) and of the context with a negative stimulus (such as a shock) can be seen as analogous and share many common features. Specifically, in contextual fear conditioning, the initially neutral context is paired with the shock and the animals learn and remember this association. When re-exposed to the context, the memory for the association can be measured by the time they freeze. In CPP, the initially neutral context is paired with the drug. The animals then exhibit conditioned drug responses, such as approaching. As such, the CPP model allows researchers to adapt the framework of established knowledge in contextual fear

memories and to compare the data in parallel, thus facilitating the understanding of drug-associated contextual memories.

There are also some technical advantages to using CPP. While repeated self-infusions of the drug is necessary to establish reliable operant behavior in the self-administration paradigm, CPP can occur following only a single context- drug pairing, such as with cocaine (Bardo, Neisewander, & Miller, 1986), morphine (Mucha et al., 1982) and amphetamine (Bardo et al., 1999).

Interestingly, even though both self-administration and CPP are two parallel paradigms that have been widely used in research, they may reflect different properties of the drug of interest. For example, buspirone induces CPP, but not self-administration and phencyclidine induces self-administration, but not CPP (Acquas, Carboni, Garau, & Di Chiara, 1990; Bardo & Bevins, 2000; Marquis Paquette, Gussio, & Moreton, 1989). Moreover, the magnitude of the amphetamine-induced CPP and the rate of self-administration do not appear to be correlated amongst individual animals (Bevins & Bardo, 1999).

1.4.4 Systems reconsolidation of drug-associated memory

One of the promising potential treatments for relapse is the "reconsolidation" stage of addiction memory (Taylor et al., 2009). Indeed, it has been reported that reactivated memories are transiently destabilized, and then restabilized (Nader et al., 2000). This destabilization/restabilization process is referred to as reconsolidation and has been studied using both aversively- as well as appetitively-motivated tasks. At the cellular level, destabilization is thought to involve protein degradation and restabilization is thought to require protein synthesis (Nader et al 2000). Importantly, reconsolidation may also occur at the systems level. I will focus on systems reconsolidation in this introduction, as it is the main research focus of this dissertation. Systems reconsolidation refers to the phenomenon that when a hippocampal-independent memory is reactivated, it returns to a labile status, susceptible to hippocampal disruption again (Debiec et al., 2002). Understanding the role of the hippocampus in this process may be important in developing pro-abstinence anti-relapse treatments for drug addiction. Systems reconsolidation was first studied using fear-conditioning paradigms, and I will elaborate these studies below.

At the cellular level, Nader et al. (2000) first reported that the consolidated fear memory becomes susceptible to protein inhibitors after retrieval. In this study, infusion of anisomycin into the amygdala locally immediately after the retrieval of the fear memory impaired fear conditioning on subsequent tests. Along with many other studies, it has been reported that retrieval of a memory trace can induce an additional labile phase that requires an active process to stabilize memory after retrieval. Interestingly, reconsolidation is different from consolidation, even though their mechanisms were overlapped partially (Tronson et al., 2007; Nader et al., 2009; 2010).

Reconsolidation also occurs at the systems level. As reviewed before, the memory circuit that initially depends on hippocampus will become hippocampal independent over time, a phenomenon referred as systems consolidation. Interestingly, it has been shown that when the hippocampus-independent memories are reactivated under some circumstances, the memory will become labile and susceptible to hippocampal disruption again, a phenomenon referred to as systems reconsolidation (Debiec, 2002). In Debiec's study, the contextual fear memory was reactivated 45 days after training (when the memory was already hippocampus-independent), and then post-activation (up to 2 days) intra-hippocampal anisomycin infusion impaired subsequent memory retrieval. In line with the systems reconsolidation hypothesis, Winocur et al. reported that only when the memory regained its context specificity through reminding, the contextual fear conditioning memory became sensitive to hippocampal disruption (Winocur et al., 2009).

Memory reconsolidation has been demonstrated not only in contextual fear conditioning, but also in appetitive conditioning (Bernardi, Lewis, Lattal, & Berger, 2009; Bernardi et al., 2006; Diergaarde et al., 2008; Lee et al., 2005; Milekic, Brown, Castellini, & Alberini, 2006; Miller & Marshall, 2005; Wells et al., 2011). At the cellular level, Miller and Marshall (2005) reported that infusions of memory pathway blockers (MEK inhibitors, U0126 and PD98059) in the nucleus accumbens core impaired the retrieval and reconsolidation of cocaine CPP memory as well as the activation of ERK, CREB, Elk-1, and Fos. Even two weeks after infusion of U0126 after the retrieval test, rats displayed no evidence of memory for their previous preference.

Another study focusing on the reconsolidation of morphine CPP is more relevant to systems reconsolidation. Milekic et al. (2006) reported that an established morphine CPP memory in rats

was persistently disrupted after reactivation of the conditioning experience (i.e., drug + context reminders) 1 week after training. Specifically, they concluded that an established morphine CPP could be disrupted if protein synthesis was blocked selectively in the hippocampus, basolateral amygdala, and nucleus accumbens, but not in the ventral tegmental area, after the reactivation. This disruption appears to be permanent. Together, these findings provide strong evidence that the reconsolidation of drug-associated memories may be a potential target for treating relapse and addiction.

A key question about systems reconsolidation is that as the memory transforms from a stable to a labile state after a reminder, does the quality of this memory change? In the standard view of systems consolidation, the remote memory represented in the extra-hippocampal structure is identical to the initial memory formed in the hippocampus. That is, the hippocampus is no longer required to retrieve the original memory (Dudai, 2004; Squire & Alvarez, 1995). The transformation theory, on the other hand, states that when the memory was transformed from the hippocampal to extra-hippocampal structures, the quality of the memory also transformed from a highly detailed and context specific form to a more schematic and context-generalized memory (Moscovitch et al., 2005; Winocur et al., 2009; Winocur et al., 2007). In other words, the expression of the original memory in its precise form may always require the hippocampus. However, to this date, it is still not clear how drug-associated memory (such as CPP memories) undergoes systems reconsolidation and whether the data from reconsolidation experiments are in line with either the standard theory or the multiple trace theory.

Even though CPP is an appetitive discriminative paradigm, previous studies have used an aversive (i.e. fear) discriminative paradigm to explore such questions. With contextual fear discrimination paradigm, Wang et al. (2009) demonstrated that although the precise context memory does not require the hippocampus at remote time points, the hippocampus might still be required to revise the existing memory trace in extra-hippocampal networks. In this study, mice were trained for three consecutive days in context A (paired with shock) and context B (never paired with shock) (Wang et al., 2009). This protocol is very similar to CPP training protocol, in which mice are trained in context A (paired with cocaine) and context B (paired with saline) and are then expected to differentiate between the two contexts. Importantly, as previously discussed, the context discrimination component may be better to address the time-dependent change in memory quality. Wang et al. (2009) showed that hippocampus lesioned mice could still

discriminate between the contexts, suggesting that the expression of the precise context memories does not require the hippocampus at remote time points. However, the observed fragility of this remote context memory also led to the hypothesis that the hippocampus may be involved in the integration of new information into existing extra-hippocampal networks. Therefore, in the present study, we will first exam the systems reconsolidation in cocaine CPP, and further explore how context specificity would affect the involvement of hippocampus, hoping to deeper our understanding for the systems consolidation process of cocaine CPP.

1.4.5 MEF2 and drug-associated memories

1.4.5.1 Drug-associated memory and drug-induced structural changes

As reviewed before, drug-associated memories play an important role in addiction, especially in relapse when contexts and cues in drug-associated memories trigger addiction behaviors in both human and animal (Muller, 2013). Indeed, more and more studies have revealed that various memory systems (such as hippocampus and amygdala) are involved in the establishment of addiction (Hyman et al., 2006; Muller, 2013). This study sets out to explore the role of structural changes in the formation of drug-associated memories.

Structural changes, synaptic plasticity for instance, have been widely studied for its role in the development memories. Bailey and Kandel (1993) first demonstrated that a simple learning process could induce weakening or strengthening of synaptic connections. Many studies later have shown the role of synaptic plasticity in memory formation. Engert and Bonhoeffer (1999) showed that new spines were found only in stimulated dendritic segments and not in unstimulated ones, directly demonstrating that plasticity can induce formation of novel spines (Engert & Bonhoeffer, 1999). Further studies have suggested that increases in spine density are important in several behavioral models of learning and memory in rodents, including spatial memory (Martin, Barad, & Kandel, 2000), bird song learning (Airey, Kroodsma, & DeVoogd, 2000), associative learning (Leuner, Falduto, & Shors, 2003), olfactory learning (Knafo, Ariav, Barkai, & Libersat, 2004), and motor learning (Yang et al., 2009; Xu et al., 2009). Interestingly, while this increase in spine density seems to be transient in the hippocampus (Eyre, Richter-Levin, Avital, & Stewart, 2003; O'Malley, O'Connell, C. Murphy, & Regan, 2000), learning-induced spines in cortical areas are more stable, lasting for weeks, months, and even years (Grutzendler, Kasthuri, & Gan, 2002; Holtmaat et al., 2005; Trachtenberg et al., 2002; Yang et

al., 2009; Zuo, Yang, Kwon, & Gan, 2005). Most relevant here is that synaptic plasticity in hippocampus and amygdala has been shown implicated in contextual fear conditioning and water maze memories (Cole et al. 2012), suggesting that structural changes are critical in the development of long-term memories. However, the involvement of structural changes in hippocampus in drug-associated memories is not clear.

In fact, it has been proposed that structural changes are also important in drug-associated memories. Relapse can be triggered by drug-associated cues or context even after long periods of abstinence, and increased dendritic spine density is reported in several different animal models of relapse (Robinson & Kolb, 2004). It has been hypothesized that the change in spine density in NAc neurons may mediate information processing from the limbic structures and further mediate addiction behaviors (Hyman et al., 2006). Moreover, repeated drug exposure induced synaptic plasticity and increased CREB and ΔFosB in NAc, both phenomena suggesting that the NAc is implicated in the maintenance of addiction (Nestler, 2013).

In this regard, the transcription factor myocyte enhancer factor 2 (MEF2) is of particular interest as it appears to plays a critical role in experience-dependent changes in spine density not only in NAc (Pulipparacharuvil et al., 2008) but also in hippocampus (Cole et al 2012). This thesis investigated the role of MEF2 in drug-associated memory and I will discuss MEF2 in more detail below.

1.4.5.2 MEF2 (myocyte enhancer factor 2)

Originally found and characterized in the muscle tissue, MEF2 is also expressed endogenously in the adult brain. It is recently found that MEF2 is implicated in memory processes, including synapse formation, regulation, and modification. Manipulating MEF2 has been shown to affect memory behavior bidirectionally (Barbosa et al., 2008; Flavell et al., 2006; Pulipparacharuvil et al., 2008). Moreover, the downstream target genes of MEF2 have been implicated in several neurological disorders (Greer et al., 2010), suggesting that MEF2 may be an important transcription factor involved in learning and memory (Flavell et al., 2006).

MEF2 proteins are ubiquitous but are most abundant in muscle, brain, and lymphocytes (Edmondson, Lyons, Martin, & Olson, 1994; Martin et al., 1994; Martin, 1993). It has been shown that MEF2 activates genetic programs which control cell differentiation, proliferations,

morphogenesis, survival, and apoptosis (McKinsey, Zhang, & Olson, 2002; Potthoff & Olson, 2007). Playing a critical part in development, MEF2 proteins are essential for viability, as complete knockout of the MEF2 protein produces a lethal cardiac and muscle phenotype (Lin, Schwarz, Bucana, & Olson, 1997; Naya et al., 2002).

There are four MEF2 genes in mammals: MEF2A, B, C, and D, all of which share similar structures and are evolutionarily conserved. Importantly, high homology in MEF2 proteins has been observed across species (Breitbart et al., 1993; Dichoso et al., 2000; Leifer et al., 1993; Lilly, Galewsky, Firulli, Schulz, & Olson, 1994; Nguyen, Bodmer, Abmayr, McDermott, & Spoerel, 1994; Rescan, 2001; Spring et al., 2002). Though they share a similar structure, the four MEF2 proteins have distinct but overlapping expression patterns during embryogenesis and in adult tissues (Lyons, Micales, Schwarz, Martin, & Olson, 1995), serving different functions in various tissues (Lin et al., 1997; Naya et al., 2002). All four proteins are expressed in the cortex and the olfactory bulb throughout development and into adulthood (Lyons et al., 1995). MEF2A is expressed in the cortex, olfactory bulb, and internal granular layer of the cerebellum, highly correlated with the expression of granule neuron differentiation markers (Lin, Shah, & Bulleit, 1996). In contrast, MEF2B is mainly expressed in the developmental stage, with limited expression in the olfactory bulb, cortex, and dentate gyrus during adulthood (Lyons et al., 1995). MEF2C, as the most extensively characterized MEF2 protein, is abundantly expressed in the adulthood brain (Lyons et al., 1995), but not expressed during development (Gong et al., 2003; Leifer, Golden, & Kowall, 1994; McDermott et al., 1993). Finally, MEF2D is primarily expressed in the developing central nervous system and throughout adulthood (Lyons et al., 1995). The entire MEF2 family share near identical N-termini, containing domains for DNA binding, dimerization, and co-factor interaction (Black, Molkentin, & Olson, 1998; McKinsey et al., 2002). On the other hand, the MEFs family has divergent C-terminus, which contains the transcriptional activation domain (Black et al., 1998). In the C-terminus, complex patterns of alternative splicing, usually in a tissue specific manner, give rise to a number of distinct isoforms (Martin et al., 1994; McDermott et al., 1993; Morisaki et al., 1997; Yu et al., 1992; Zhu & Gulick, 2004). In relevance to the present study, MEF2A and MEF2D are the isoforms most highly expressed in the forebrain regions, including the hippocampus (Pfeiffer et al., 2010).

MEF2 target genes are implicated in memory. Flavell et al. (2006) identified a set of activity-dependent MEF2 target genes that negatively regulate synapse formation. In response to

increased neuronal activity, calcium influx into neurons induces the activation of the calcium/calmodulin-regulated phosphatase calcineurin. Calcineurin dephosphorylated and activated MEF2, promoting the transcription of genes that negatively regulate the synapses. Arc is among these activity-dependent MEF2 target genes and essential to memory (Bramham, Worley, Moore, & Guzowski, 2008; Tzingounis & Nicoll, 2006). In fact, many of these activity-dependent MEF2 target genes have already been implicated in memory, serving roles in excitatory synapse weakening, excitatory synapse maturation, inhibitory synapse development, and presynaptic vesicle release. These genes include homer1a (Celikel et al., 2007; Lominac et al., 2005), c-fos (Guzowski, Setlow, Wagner, & McGaugh,, 2001; Stanciu, Radulovic, & Spiess, 2001), and BDNF (Greenberg, Xu, Lu, & Hempstead, 2009; Lipsky & Marini, 2007).

The effect of MEF2 itself on synapses has also been examined in vitro and in vivo. Selective activation of MEF2-dependent transcription results in rapid and robust synapse elimination, and knockdown or gene deletion of MEF2 isoforms results in increased synapse numbers in an activity-dependent manner (Barbosa et al., 2008; Flavell et al., 2006; Pulipparacharuvil et al., 2008). Furthermore, in vivo, an MEF2-dependent synapse elimination process has been shown to be critical for normal learning, memory, and behaviors associated with drug abuse (Barbosa et al., 2008; Pulipparacharuvil et al., 2008).

Collectively, MEF2 proteins have been hypothesized to be critical in memory. In the current study, we examined the role of structural changes in the hippocampus in drug-associated contextual memory. Specifically, based on the fact that over expressing MEF2 impairs contextual fear memories and water maze memories, we hypothesized that preventing structural plasticity by over expressing MEF2 in hippocampus could block the cocaine CPP memory.

2 Research Aims

In the present study, we investigate how cocaine-associated contextual memories reorganize over time. Specifically, we will address three aims:

1) To examine the effect of hippocampal lesion on recent and remote cocaine CPP memory. Based on the evidence of systems consolidation found in contextual fear memory studies (Frankland and Bontempi, 2005), we hypothesize that a recently formed contextual drug-associated memory depends on hippocampus but will become hippocampal independent over time. In parallel to hippocampus, we will also study the effect of lesions of the nucleus accumbens on expression of a cocaine CPP. Because the nucleus accumbens plays a central role in general reward and cocaine-induced behaviors, we hypothesize that lesions of the nucleus accumbens will disrupt expression of cocaine CPP at both recent and remote delays after training.

2) To examine the effect of hippocampal lesions on reactivated remote cocaine CPP memories. Previous contextual fear conditioning studies have shown that reminder treatments may make remote (hippocampus-independent) memories transiently hippocampus-dependent again (Debiec et al., 2002). We will test whether a similar process of 'systems reconsolidation' occurs for contextual drug-associated memory. Specifically, we hypothesize that hippocampus would be reengaged by exposing test animals to a reminder. We will further investigate the effect of different types of reminders (i.e., presentation of the CS together with the US, the CS alone or US alone) on systems reconsolidation, as it has been reported that different reminders induce different levels of hippocampal dependency (Winocur et al., 2009).

3) To examine the effect of synaptic structural changes in the hippocampus on cocaine CPP. Spatial and contextual fear memory formation is associated with structural plasticity (e.g., growth of spines) in the hippocampus (Cole et al., 2012), and blocking learning-related spine growth by over-expressing the transcription factor MEF2 impairs memory consolidation. Accordingly, we hypothesize that MEF2-induced synaptic changes in the hippocampus play a similar role in formation of cocaine CPP memory. To test this hypothesis, we adopt the

experimental approach of Cole et al. (2012) to overexpress MEF2 via HSV in hippocampus during and after CPP training and explore effects on CPP memory.

2.1 Aim 1: examining the systems consolidation of cocaine CPP

Systems consolidation has been demonstrated and studied in detail using the contextual fear condition paradigm. It is the phenomenon that memories reorganize over time among different brain regions; specifically, a recent contextual fear memory requires the hippocampus for its expression, whereas a remote contextual fear memory is hippocampus independent. Evidence of systems consolidation has been widely documented in human and animal literature (see review in Frankland & Bontempi, 2005; Akers & Frankland, 2009). For instance, disrupting hippocampal function preferentially impaired a recent (1 day old), but not a remote (28 day old), contextual fear memory (Kim & Fanselow 1992). Recall of a recent contextual fear memory activates hippocampus, while recall of a remote memory activates mainly cortical regions (Frankland et al. 2004; Wheeler et al. 2013).

Even though there is an abundance of evidence for systems consolidation for contextual fear memory, it remains unclear that if a similar pattern can be observed in contextual drug-associated memories. The current research investigates these questions using cocaine conditioned place preference (CPP) paradigm.

The CPP model is chosen because it shares critical common features with contextual fear conditioning. First, both paradigms are highly context dependent. Second, hippocampus appears to be critical for both paradigms. For CPP in particular, studies have suggested that pre-training hippocampal lesion impairs cocaine CPP (Meyers et al, 2003), and that hippocampal neuronal activity is increased when rodents are exposed to a previous cocaine-paired environment (Neisewander et al., 2000). Third, both paradigms generate a robust long lasting memory, and therefore memory can easily be assessed at a remote time point. Given these similarities, we hypothesize that systems consolidation will occur in CPP as well. It is also worth noting that, CPP is an appetitive paradigm and neuronal substrates involved may be different from contextual fear memory. In this sense, the current study may expand the scope of systems consolidation from aversive contextual fear memories to appetitive contextual drug memory.

To examine the systems consolidation in cocaine CPP, we adopt the experimental design of the contextual fear conditioning studies. Specifically, we will lesion the hippocampus either 1 day (a recent cocaine CPP memory) or 30 days (a remote cocaine CPP memory) after cocaine CPP conditioning. This design allows us to examine lesion effects at different time points and test whether there exists a temporal gradient of retrograde amnesia. In parallel to hippocampus, we will also lesion the nucleus accumbens as it is considered a critical brain region for general reward and cocaine-induced behavior (Baik 2013). Based on the evidence collected mainly from the contextual fear memory studies, we hypothesize that hippocampus plays a temporally-graded role in cocaine CPP. Thus, hippocampal lesion disrupts only the recent, but not remote cocaine CPP expression. In contrast, nucleus accumbens is likely to be necessary for both recent and remote cocaine CPP memory.

2.2 Aim 2: examining the systems reconsolidation in cocaine CPP

Reminder treatments may render remote, hippocampus-independent memories dependent upon the hippocampus once more for their expression (Debiec et al., 2002). Indeed, as a remote contextual fear memory becomes hippocampus-independent, it also loses its context specificity (Wiltgen & Silva 2007). However, when a context specific reminder reactivates a remote contextual fear memory, the memory regains the context specificity, and becoming hippocampus dependent again (Winocur et al., 2009).

In the current study I will address whether a systems reconsolidation process occurs for a cocaine CPP memory. In addition, this study also assesses the impact of different types reminders on hippocampus reengagement after remote memory reactivation. Specifically, mice with a 30-day old cocaine CPP memory will be exposed to various reminders (i.e., presentation of the CS together with the US, the CS alone or US alone). One day after the exposure, hippocampal lesion surgeries will be conducted on these mice. We hypothesize that subsequent cocaine CPP memory will not be affected in the no-reminder group. In contrast, following reminders, remote cocaine CPP memories will become sensitive to the effects of hippocampal lesions. These effects may vary as a function of reminder strength.

2.3 Aim 3: examining the role of structural changes within the hippocampus in the formation of cocaine CPP memory

It has been proposed that memory and addiction induces long-term neuronal structural changes in the brain, and these structural changes are thought to underlie memory formation, storage, and relapse (Hyman, 2005). Recent studies found that MEF2 restricts dendritic spine growth in an activity-dependent way (Flavell et al., 2006), suggesting that MEF2 may suppress memory formation. Importantly, locally and acutely increasing MEF2 function in hippocampus impairs spatial memory formation; increasing MEF2 function in amygdala blocks fear memory formation (Cole et al., 2012). Moreover, Pulipparacharuvil et al. (2008) have also shown that overexpressing MEF2 in nucleus accumbens inhibits cocaine-induced spine increase and lead to increased sensitivity to cocaine as well as increased cocaine CPP. However, it is still unknown if the MEF2-induced structural changes in hippocampus is involved in the formation of cocaine CPP memory. Toward this end, we adopt the experimental approach of Cole et al. (2012) and overexpress MEF2 acutely and locally in hippocampus to examine the role of structural changes in hippocampus in cocaine CPP memory.

We will use the HSV viral vector system to acutely and locally overexpress MEF2 in hippocampus during- and post- conditioning. Based on the evidence found in spatial and contextual fear memories, we hypothesize that overexpression of MEF2 in hippocampus would limit the development of cocaine CPP memory.

3 Materials and Methods

3.1 Subjects

Mice were bred in the colony at The Hospital for Sick Children and maintained on a 12 hr light/dark cycle (lights on at 0700 hrs). Female hybrid offspring (C57B1/6 x 129Svev) from a cross between C57Bl/6NTacfBr [C57B6] and 129Svev [129] mice (Taconic, Germantown, NY) were used for all experiments. All mice were bred in the colony room at Hospital for Sick Children, and were maintained on a 12 h light/dark cycle with free access to food and water. Behavioral experiments were conducted during the light phase of the cycle. Mice were at least 8 weeks old at the time of training. Experiments were conducted by the experimenter who was blind to the treatment condition of the mouse. All experimental protocols were approved by the Animal Care Committee at The Hospital for Sick Children.

3.2 Apparatus

3.2.1 CPP apparatus

CPP was conducted in a custom made Plexiglas chambers (20cm X 45cm X 20cm) (See Figure 3.1.) The chamber could be divided into two compartments of equal size, separated by a removable divider. Each compartment had distinct visual, tactile and olfactory cues. The white compartment had white walls, rough flooring and scentless. The stripe compartment had black and white stripe walls, smooth flooring, and a vinegar scent (0.2 ml 3% acetic acid) applied prior to conditioning and test session. In our pilot study, mice showed no baseline preference for either compartment. Moreover, in all experiments, a counterbalanced design was used. That is, half the mice were cocaine conditioned in the white side, while the other half were cocaine conditioned in the stripe side. Mouse movement was tracked and recorded automatically with a video camera interfaced to the Limelight Video Tracking software (Coulbourne Instruments).

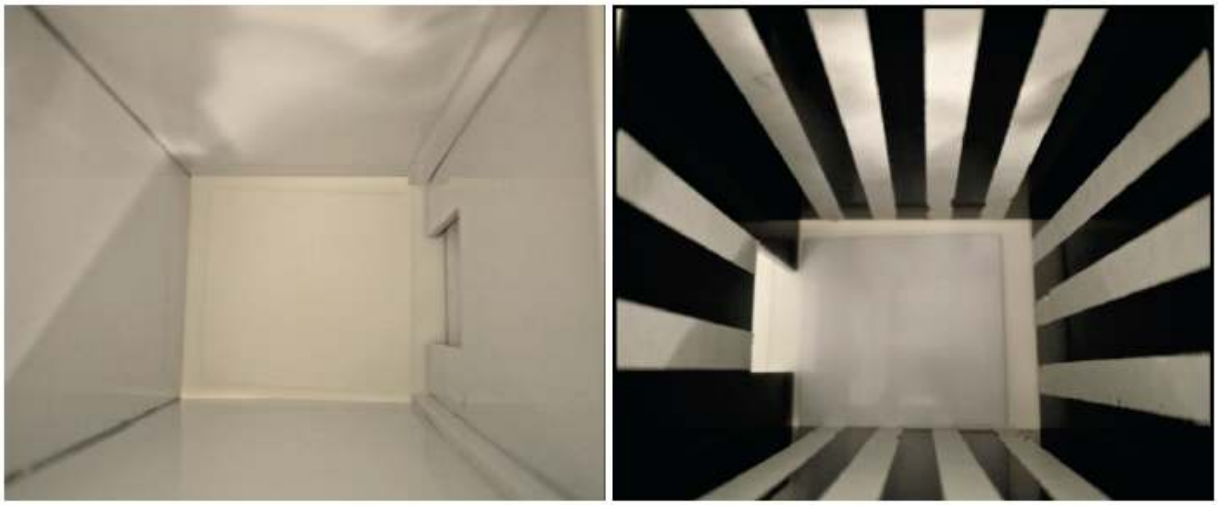

Figure 3.1 CPP Apparatus

3.2.2 CPP procedures

3.2.2.1 Pre-conditioning

For all experiments, mice were transferred into the colony room at least 2 weeks prior to conditioning to minimize anxiety caused by transportation. Mice were handled for four days, four minutes per day prior to conditioning.

3.2.2.2 Conditioning

In the lesion studies, six conditioning sessions were conducted for 6 consecutive days. On days 1, 3 and 5, mice were injected with cocaine (12 mg/kg, i.p.) and confined in the drug-paired compartment for 15 min. On day 2, 4, and 6, mice were injected with the same volume of saline and confined to other compartment for 15 min. During the conditioning session, the distance mice travelled were measured.

In the MEF2 studies, four conditioning sessions were conducted over 2 consecutive days. Mice were conditioned once in the morning and a second time in the afternoon, separated by at least 6 hours. In the morning, mice were injected with saline and confined in the saline-paired compartment for 15 min. In the afternoon, mice were injected with same volume of cocaine (15 mg/kg, i.p.) and confined in the other compartment for 15 min.

3.2.2.3 Test

During the test phase, mice had free access to both compartments for 10 min under drug free conditions. Saline was injected i.p. immediately before the animals were placed in the apparatus. The locomotion path was traced and the amount of time spent in each compartment was measured.

3.2.3 Water maze apparatus

The circular water maze tank (120 cm in diameters, 50 cm deep) was located in a dimly lit room (Teixeira, Pomedli, Maei, Kee, & Frankland, 2006). The pool was filled to a depth of 40 cm with water made opaque by adding nontoxic white paint. The water temperature was maintained at 28 ± 1°C by a heating pad beneath the pool. A circular escape platform (10 cm diameter) was submerged 0.5 cm below the water surface and located in one of the quadrants in a fixed position throughout training (target quadrant). The pool was surrounded by white curtains at least 1 m from the perimeter of the pool. The curtains were white and had distinct cues painted on them. Behavioral data from training and the probe tests were acquired and analyzed using an automated tracking system (Actimetrics, Wilmette, IL). This software allowed for parameters to detect escape latency and swim speed to be recorded during training. In probe test, the percent of time mice searched the target zone (20 cm in radius, centered on the location of the platform during training) versus the average of three other equivalent zones in other areas of the pool was measured. Each of the zones represents approximately 11% of the total pool surface.

3.2.4 Water maze procedures

3.2.4.1 Pre-training

Mice were transferred into the colony room at least 2 weeks prior to training to minimize anxiety. Mice were then handled for seven days, 2 minutes per day prior to the training. For the remote and no reminder groups, water maze was performed after CPP therefore there was no additional handling before water maze training.

3.2.4.2 Training

Mice received 6 training trials (presented in 2 blocks of 3 trials, with the intertrial interval of 15 seconds, and an interblock interval of 1 hour). At the start of each trial, mice are placed on the

platform for 15 sec. On each trial, mice were placed in the pool, facing the wall in one of four start locations. The order of the start locations was pseudorandomly varied. The trial was complete once the mouse found the platform or 60s had elapsed. If the mouse failed to find the platform on any trial, the experimenter guided the mouse onto the platform. Behavioral data from training were acquired and analyzed using an automated tracking system (Actimetrics, Wilmette, IL).

3.2.4.3 Test

Sixty minutes following the last training trial, spatial memory was assessed with a probe test. During the probe test, the platform was removed from the pool and the mice were given 60 seconds to search the pool. Behavioral data from probe tests were acquired and analyzed using an automated tracking system (Actimetrics, Wilmette, IL). Time spent in different zones was recorded, including the target zone where the platform located in the training trial. The percentage of time mice spent searching in the target zone versus the three other zones was measured.

3.3 Stereotaxic lesions

3.3.1 Surgical procedures

3.3.1.1 Hippocampal lesion procedures

Mice were pre-treated with atropine (5 mg/kg, i.p.) and anesthetized with chloral hydrate (10 mg/kg, i.p.). In order to prevent potential seizures associated with neurotoxic lesions, mice were additionally pretreated with diazepam (5 mg/kg, i.p., Sigma, St. Louis, MO). Using standard stereotaxic procedures, N-methyl-D-aspartic acid (NMDA, 10 mg/ml; Sigma, St. Louis, MO), the agonist at the NMDA receptor, was infused into the following sites with respect to bregma: for hippocampus: AP -2.00, ML ±1.50, DV 1.80 (volume=0.12 µl); AP -2.50, ML ±1.8, DV 2.00 (volume=0.12 µl); AP-3.06, ML ±2.75, DV 3.50 → 3.00 (volume=0.25 µl) according to the mouse brain atlas (Paxions & Franklin, 2001). NMDA was delivered via a 32-guage injection needle connected to a Hamilton microsyringe (Hamilton, Reno, NV). An infusion pump maintained the rate of infusion at 0.1 µl /min and the injection needle was left in place for 5

minutes following the completion of the infusion. For sham surgeries, mice were treated identically except no NMDA was infused. Mice were treated post-operatively with the analgesic ketoprofen (5 mg/kg, ip, Sigma, St. Louis, MO). The procedures are based on previously published reports using NMDA to lesion hippocampus (Wang et al., 2009; Deacon & Rawlins, 2005; Deacon, Bannerman, Kirby, Croucher, & Rawlins, 2002.)

3.3.1.2 Nucleus accumbens lesion procedures

Mice were treated as above but the NMDA was injected into the nucleus accumbens at the following coordinates: AP+1.34, ML ±0.80, DV 4.50 (volume=0.15 µl) relative to bregma (Paxions & Franklin, 2001).

3.3.1.3 MEF2 surgical procedures

Using the standard stereotaxic procedures described above, MEF2-VP16 viral vector (or GFP control) were administered bilaterally in a volume of 2.0 µl per side at a rate of 0.1µl/min for 20 min using glass micropipettes attached to a 10 µl Hamilton syringe. After infusion, the pipette was left in place for 5 min to allow for diffusion. The coordinates for the infusion were AP: -2.0, ML: ± 1.0, DV: - 2.1, relative to bregma (Paxions & Franklin, 2001). Mice were treated post-operatively with the analgesic ketoprofen (5 mg/kg, i.p.; Sigma). Surgery protocols are based on previously published studies using similar viral vector and infusion methods (Han et al., 2009).

3.3.2 Histology

3.3.2.1 Hippocampal lesion histology

At completion of behavioral experiments, all animals were perfused transcardially with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. The brains were removed and post-fixed for 24 hr in PFA. Brains were then transferred and stored in 30% sucrose solution for at least 48 hr before sectioning. The brains were sectioned in the coronal plane to 50 μm thick using a cryostat microtome at -19 °C. The entire anterior-posterior extent of

the hippocampus were sectioned and subsequently stained with neutral red for visualization of hippocampal lesion. Sections were mounted on slides (VWR, West Chester, PA) with Permafluor anti-fade medium (Lipshaw Immunon, Pittsburgh, PA). The hippocampus was anatomically defined according to a mouse brain atlas (Paxions & Franklin, 2001).

Quantification of lesions

Detailed quantification of neuronal loss was conducted on neutral red-stained sections. The extent of brain damage in the lesion and sham mice was evaluated by another experimenter, who was blind to the group assignment. Briefly, at 10X magnification, the area of hippocampal subregions (CA fields and dentate gyrus) containing neutral red+ cells was estimated using ImageJ (NIH) from anterior (dorsal) to posterior (ventral) extent of the hippocampus. Mean areas for the 3 most anterior (dorsal) and 3 most posterior (ventral) levels were then calculated for each mouse, and these values were averaged across animals. The inclusion/exclusion criteria were as followings: First, the lesion must be largely confined to the hippocampus, with limited damage to surrounding tissue. Second, lesion in the hippocampus should be bilateral and present in both anterior and posterior portions for over 80% of total damage. Due to the nature of excitotoxic lesions, some mice had been euthanized during the post-surgical recovery period. No behavioural data was included for the euthanized animals.

3.3.2.2 Nucleus accumbens lesion histology

The histology procedures for nucleus accumbens were identical to the procedures for hippocampus. The inclusion/exclusion criteria for nucleus accumbens were as follows: First, the lesion must be largely confined to the nucleus accumbens, with limited damage to surrounding tissues. Cell loss or cell disorganized was restricted to the nucleus accumbens core and shell, with minimum damage to other brain regions. In the region of lesions, neurons have been replaced by disorganized and loosely-staining cells. Neuronal loss extended in an antero-posterior direction from approximately 2.0 mm to 0.6 mm anterior to bregma, and did not extend ventrally or caudally into the ventral pallidum or olfactory tubercle. Some small local damage around the needle tracts could be seen in the cortex overlying the nucleus accumbens in both

sham and lesion groups. Second, lesions in the nucleus accumbens should be bilateral and present in over 80% areas from anterior to posterior portions of nucleus accumbens.

3.3.2.3 MEF2 histology

At completion of the behavior experiments, all animals were perfused as described above. The entire anterior-posterior extent of the hippocampus were sectioned and subsequently mounted on slides using Vectashield mounting medium with DAPI for visualization of viral expression. Sections were analyzed under an epifluorescent microscope to confirm infusion placements using the expression of GFP (presented in both viruses). The inclusion/exclusion criteria were as followings: First, the viral expression must be largely confined to the dentate gyrus of hippocampus. Second, viral expression in the hippocampus should be bilateral and present in anterior and posterior portions.

3.4 Experimental procedures

3.4.1 Experiment 1: systems consolidation of CPP

Mice in both recent and remote groups were trained using the CPP procedures described in Section 3.2.2, and then were randomly assigned into lesion or sham group. Either one day (in recent group) or thirty days (in remote group) after conditioning, mice received surgery (either lesion or sham) as desrbed in Section 3.3.1.1. After 10 days of recovery, mice were put back to the standard CPP apparatus with free access to both cocaine- and saline-paired compartments and the time spent in each compartment was recorded by Limelight Video Tracking software.

3.4.2 Experiment 2: reengagement of hippocampus in the remote cocaine CPP memory after reactivation

In the next experiment, the reengagement of the hippocampus in remote CPP memory retrieval was assessed by the presenation of different reminders 1 day prior to lesion surgery.

All mice were conditioned for cocaine CPP as previously described in Section 3.2.2, and were then randomly assigned into different groups. Different reminders (or no reminder) were given one day prior to the lesion surgery, which was 30 days after training. For example, in context only group, mice were put back into the cocaine-paired context for 5 minutes as a context reminder. In drug alone group, mice were injected with cocaine (12mg/kg, i.p.) in their homecage as a drug alone reminder. In drug+context group, mice were injected with cocaine (12mg/kg, i.p.) and then confined in the cocaine-paired context for 5 min as a drug+context reminder. After 10 days of recovery from surgerical operation, mice were tested in the standard CPP aparatus with free access to both cocaine- and saline-paired compartments and the time spent in each compartment was analyzed.

3.4.3 Experiment 3: MEF2 and conditioned place preference

3.4.3.1 MEF2 Virus

In this study, a replication-defective herpes simplex virus (HSV) was used to overexpress MEF2. We chose to use the HSV viral systems because it infects mature neurons, rather than glia. Secondly, the DNA from HSV remains episomal and would not cause mutagenesis by inserting genes into the host DNA (R. Neve, K. Neve, Nestler, & Carlezon Jr, 2005).

For our study, p1005(+) amplicon backbone was used. This backbone has a multiple cloning site where the gene of interest is driven by the HSV immediate-early gene IE4/5 and the GFP is driven by a human cytomegalovirus immediate-early gene (CMV) promoter (Figure 3.2). In this way, GFP can be used as a marker of infected neurons. To increase MEF2-dependent transcription, we expressed MEF2-VP16, a version of MEF2 in which the DNA binding and dimerization domains are fused to the transcriptional activation domain of the viral protein VP16. MEF2-VP16 binds MEF2 sites within the promoter region of target genes and leads to their constitutive transcription. Also, p1005(+)-GFP without any additional transgene was used as a control vector. This vector expresses GFP only. The average titer of the recombinant virus stocks was typically $4.0x10^7$ infectious units/ml. Transgene expression peaks 2-5 days following infusion and declines within 7 days (Vetere et al., 2011).

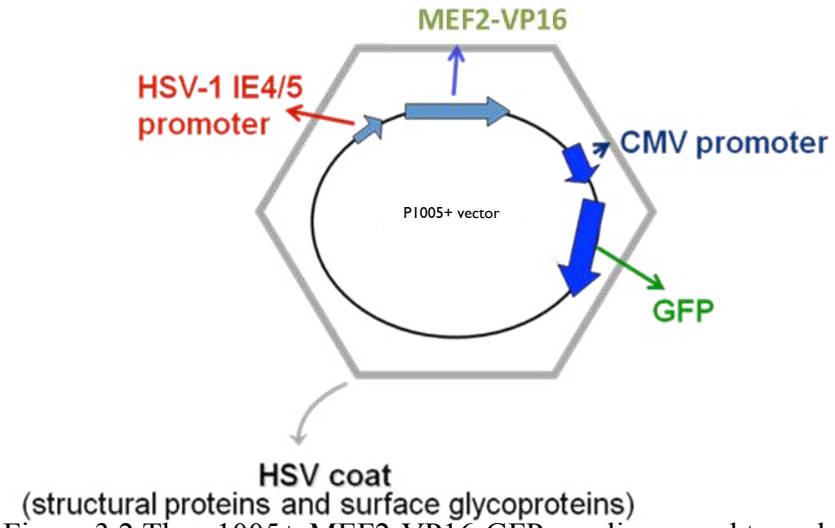

Figure 3.2 The p1005+-MEF2-VP16-GFP amplicon used to make viral vectors.

The amplicon contains MEF2-VP16 cDNA and green fluorescent protein (GFP). MEF2 expression is driven by the HSV immediately early (IE) 4/5 promoter and GFP expression is driven by the human cytomegalovirus immediate-early gene (CMV) promoter.

3.4.3.2 MEF2 CPP experimental procedures

All mice were trained with the modified two-day CPP protocol (modified from Section 3.2.2). To overexpress MEF2 during CPP training, HSV expressing MEF2 or GFP control was infused into dentate gyrus (Section 3.3.1.3) two days prior to the conditioning. Mice were conditioned in the morning and afternoon for two days. In the post-training group, HSV expressing MEF2 or GFP control was infused into dentate gyrus (Section 3.3.1.3) one day after CPP conditioning. After seven days of recovery, mice were put back to the standard CPP apparatus with free access to both cocaine- and saline-paired compartments and the time spent in each compartment was measured.

3.5 Statistics

3.5.1 Statistics for CPP data

To evaluate CPP memory, time spent in each of the CPP compartments during the test were analyzed with the *STATISTICA* program using two-way analyses of variance (ANOVA) and

Student's *t*-test for pairwise comparison. Effects were considered significant when $P < 0.05$. Specifically, for lesion studies, an ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Side* (time spent in cocaine-paired side versus time spent in saline-paired side) as a within-subject factor was conducted on the data. To evaluate the preference directly, time spent in cocaine-paired versus saline-paired sides was compared using paired *t*-tests in both lesion and sham groups. For MEF2 studies, an ANOVA with *Vector* (MEF2 versus GFP) as a between-subject factor and *Side* (time spent in cocaine-paired side versus time spent in saline-paired side) as a within-subject factor was conducted on the data. Paired *t*-tests were also used to further assess the difference in both groups. To assess whether mice had hyperactive behaviors during test, locomotor activity was analyzed by using unpaired t-tests for comparing the distance travelled on test day.

3.5.2 Statistics for water maze data

Escape latency during training and time spent in target zone or other zones during the probe test were analyzed with the *STATISTICA* program using two way (ANOVA). Significant main effects or interactions were followed up with Bonferroni post-hoc tests, where appropriate. Effects were considered significant when $P < 0.05$. Specifically, for comparing the escape latency between groups, an ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Time* (days of training) as a within-subject factor was conducted on the data. For probe tests, to evaluate whether mice searched selectively in the target zone, an ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Zone* (target versus other) as a within-subject factor was conducted.

In the first series of experiments, we asked whether the neural circuits supporting CPP memory change with time after its formation. First, we investigated whether the hippocampus is essential at different time points after CPP training. Specifically, we examined three different time points:

1) Recent CPP memory: Mice were given hippocampal lesions 1 day after cocaine CPP training (i.e. 1 day old).

2) Remote CPP memory: Mice were given hippocampal lesions 30 days after cocaine training. Between training and lesion surgery, mice remained in their homecages.

3) Remote CPP memory with reminders: Mice were given hippocampal lesions 30 days after cocaine training, but 1 day before the hippocampal lesion surgery, mice were given a brief reminder of CPP. Therefore, in this condition, mice were lesioned at a time when CPP memory was remote, although this memory was reactivated recently (1 day prior to surgery).

We next examined the impact of nucleus accumbens lesions at different these time points after formation of a cocaine CPP memory.

In the third series experiments, we explored how structural plasticity in hippocampus affects the formation of new cocaine CPP memory. We used a viral vector to overexpress the transcription factor Myocyte Enhancer Factor 2 (MEF2) locally in the dorsal hippocampus prior training. We found that hippocampal overexpression of MEF2 before training impaired the formation of cocaine CPP memory. However, overexpression MEF2 after training did not disrupt cocaine CPP, once established.

Our overall findings reveal that the hippocampus plays a critical role in the formation and consolidation of new cocaine CPP memory, and that MEF2 may be a key regulator underlying this.

4 Results

In the first series of experiments, we asked whether the neural circuits supporting CPP memory change with time after its formation. First, we investigated whether the hippocampus is essential at different time points after CPP training. Specifically, we examined three different time points:

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Our overall findings reveal that the hippocampus plays a critical role in the formation and consolidation of new cocaine CPP memory, and that MEF2 may be a key regulator underlying this.

4.1 Optimizing cocaine-induced CPP in mice and NMDA-induced excitotoxic lesion procedures

4.1.1 Optimizing cocaine CPP model in our hybrid mice

The first series of experiments were conducted to establish the optimal cocaine CPP training protocol for our hybrid mice (C57B1/6 x 129Svev as defined in the materials and methods). These experiments had three main goals:

1) We wanted to use an unbiased CPP protocol in which naïve mice did not exhibit pre-training preference for either the drug-paired side or saline-paired side of the apparatus. For example, one of the problems we encountered is that mice demonstrated natural preference for the darker environment. To solve this problem, acetic acid (0.2 ml 3%) was used to balance the darker environment. Also, the lighting in the room was dimmed such that mice could freely explore the environment in a less anxious state. Mice were also handled before training to reduce anxiety levels.

2) Stable and robust cocaine CPP should be observed. We examined the impact of cocaine dose on the amount of preference produced. We compared the CPP induced by several dosing strategies and settled on a 6-day training protocol using 12mg/kg cocaine.

3) Stable and robust cocaine CPP should be observed 30 d following training. Our pilot training protocols varied from 1 day to 14 days and produced different levels of cocaine CPP. We chose a 6 day/6 pairing protocol, which produced stable long-lasting cocaine CPP in our mouse strain.

The following experiments were used to optimize the CPP protocol.

Pre-conditioning Test

A pre-conditioning test (pre-test) in which mice had access to both sides of the CPP apparatus was performed to test the initial side preference (see Materials and Methods). During the pre-conditioning phase, mice (n=8 per group) showed no initial baseline preference for either to-be-paired-with saline or cocaine compartment (Figure 4.1B). Paired t-test was performed on the data and no statistically reliable difference was observed between compartments was observed (paired

t-test [$t(7)= -0.26$, $P >0.05$]). Therefore, mice have no pre-training preference for either compartment of the apparatus.

Because pre-conditioning exposure to the conditioning context has been shown to confound the effect of conditioning (Bardo & Bevins, 2000), we did not include the pre-conditioning test phase in our subsequent experiments. To further make sure that both groups of mice were trained in a counterbalanced way, the saline-paired and drug-paired side were equally assigned to both compartments of apparatus in both groups.

Training

All mice were trained for 6 consecutive days. On days 1, 3 and 5, mice were injected with cocaine (12 mg/kg, i.p.) and confined in the cocaine-paired compartment for 15 min. On days 2, 4, and 6, mice were injected with same volume of saline and confined in the other compartment. All mice showed increased locomotion on cocaine-paired days compared to saline-paired days. The average distances travelled on cocaine-pairing days and on saline-pairing days was calculated. A paired t-test was performed on the data, and we found a significant difference between cocaine and saline days [$t(7)= 6.73$, $P <0.05$] (Figure 4.1C), indicating that the cocaine increased mice locomotion during training.

Test

On test day, mice were returned to the original context with free access into both saline-paired and cocaine-paired compartments. We observed a significant increase in time spent in the cocaine-paired side versus saline-paired side. A paired t-test was performed on the time mice spent during the test, and showed significant difference between the saline-paired and drug-paired side (paired *t*-tests [$t(7)= 9.41$, $P <0.001$]) (Figure 4.1D). This data suggest that with this protocol, mice formed robust cocaine CPP. To ensure that this cocaine CPP memory lasts for 30 days, we re-tested these mice again thirty days after training. A significant increase in time spent in the cocaine-paired side versus saline-paired side was still observed [$t(7)= 4.00$, $P <0.05$], suggesting that mice formed a long-lasting cocaine CPP with this protocol.

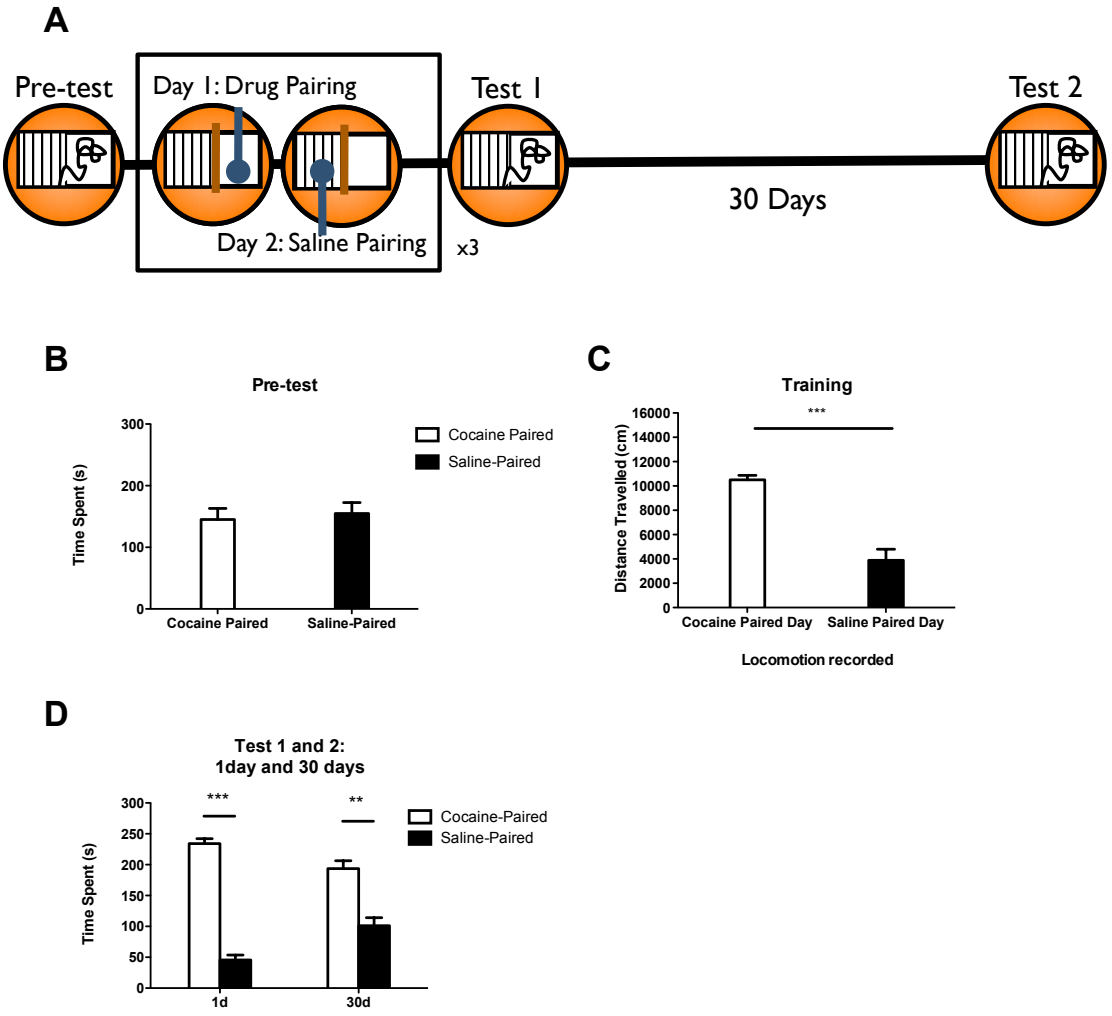


Figure 4.1 Optimization of cocaine-induced CPP task in our C57B1/6 x 129Svev hybrid mice.

Experimental design. Pre-test: Naïve mice were given a pre-test before training to measure the pre-training preference for the to-be-paired with saline and cocaine sides. Training: 3 cocaine and saline pairings were given. Test 1: 1 day after training, mice were given free access to both sides of the apparatus to test their place preference. Test 2: Mice were put back to their homecage and 30-days later, tested again.

A. Experimental design

B. Pre-test place preference for the cocaine-paired and saline-paired side. Bars represent the time (seconds) mice spent in each side during a 10-minute test session. No difference was found, indicating that mice showed no baseline preference for either compartment.

C. Cocaine-increased locomotion during training. Bars represent the average distance traveled during the 15-minute training session. Significantly increased locomotion was found in the cocaine-pairing day, indicating that activity was increased during the

cocaine-paired days. This finding replicates pervious data showing that cocaine increases locomotion during training.

D. Post-training place preference for the cocaine-paired and saline-paired side. Bars represent the time (seconds) mice spent in each side from a 10-minute test session. In both tests (day 1 and day 30), mice spent significantly more time in the cocaine-paired side, indicating that this CPP training protocol produced a long-lasting cocaine CPP memory (at least 30 days). Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by paired t-test.

4.1.2 The NMDA-induced excitotoxic hippocampal lesions in hybrid mice

The second series of experiments were conducted to validate that NMDA-induced excitotoxic lesions of the hippocampus produce spatial memory deficits in our mice. Details can be found in the material and methods chapter. Briefly, for these lesions, our inclusion criteria were:

1) The extent of the damage must be largely confined to the hippocampus.
2) At least 80% of the hippocampus must be lesioned.

Previous studies have shown that mice with hippocampal lesions have deficits in both acquisition and expression of spatial memory as assessed in the water maze (Morris, Garrud, Rawlins, & O'Keefe, 1982). Therefore, we used water maze test to validate our NMDA-induced hippocampal lesion in hybrid mice.

Water maze

To verify the effectiveness of our lesion, we lesioned the hippocampus (or performed sham surgery) in 18 mice prior to training in the water maze task. In this task, mice are trained to locate a submerged platform based on spatial cues. We trained mice for 11 consecutive days. To assess memory formation, probe tests were conducted on alternative days after training (i.e. on day 1, 3, 5, 7, 9 and 11). During a probe test, the platform was removed from the pool and mice allowed to search for it.

Over the course of training, sham-lesioned mice showed a decreased latency to find the submerged platform whereas lesioned mice did not. The average latency to find the hidden platform was significantly higher in lesioned mice. An Analysis of Variance (ANOVA) was conducted on the latency scores over training with *Surgery* (lesion versus sham) as a between-subject factor and *Time* (11 days) as a within-subject factor. A significant interaction between *Surgery* x *Time* [$F(1,10)=2.22$, $P<0.05$] as well as the significant main effects of surgery and time [*Surgery* $F(1,17)=33.26$, $P<0.001$; *Time* $F(1,10)=12.06$, $P<0.001$] were observed. Newman-Keuls post-hoc analysis showed that lesioned mice found the platform much slower than sham-lesioned mice. As we expected, these statistical findings indicated that sham mice acquired water maze task faster than the lesioned mice (Figure 4.2A).

Lesioning the hippocampus also produced robust spatial memory effects as measured during the probe tests. The amount of time mice spent in the target zone (20 cm radius circular zone centered on the former platform location) was used as an index of water maze memory. A high amount of time spent in the target zone reflects robust spatial memory. We observed that sham mice gradually formed a spatial memory over training days, but the lesioned mice did not. An ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Time* (6 probe tests) as a within-subject factor was conducted on the data. This analysis revealed a significant interaction between *Surgery* x *Time* as well as main effects of both *Surgery* and *Time* (*Surgery* x *Time: F*[1,5]=11.30, *P*<0.001; *Surgery: F*(1,17)=36.43, *P* <0.001; *Time: F*(1,5)=26.06, *P*<0.001;Figure 4.2), indicating that, over training days, sham mice spent more time in target zone than lesioned mice. Collectively, the data showed that our NMDA-induced excitotoxic lesion effectively disrupts spatial memory formation as assessed in the water maze, a classic hippocampus-mediated task (Morris et al, 1982).

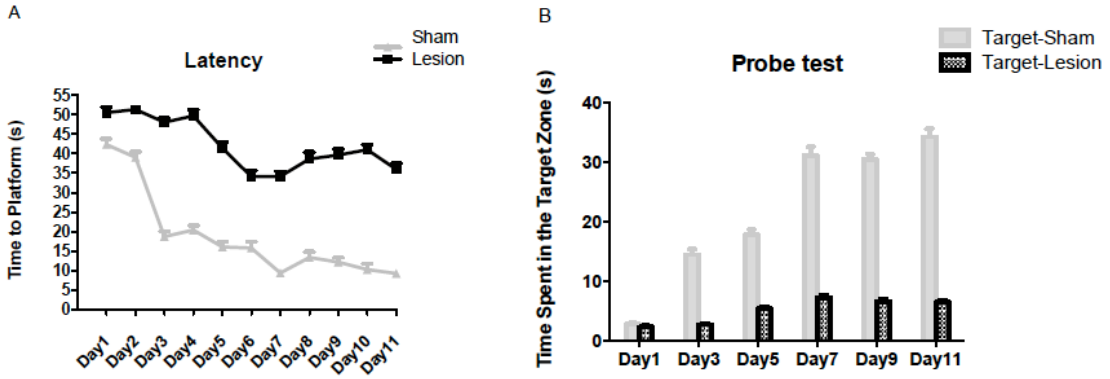


Figure 4.2 Using water maze task to validate our NMDA-induced excitotoxic lesions of the hippocampus: we observed impaired spatial memory in lesioned mice.

(A) Latency to reach the hidden platform during training decreased over training days in sham- but not hippocampal-lesioned mice. The lines represent the time (seconds) needed for mice to find the hidden platform. As showed in solid black line, lesioned mice took more time to reach the platform than sham mice (grey line), indicating that lesioning the hippocampus produces deficits in learning the location of the platform ($P < 0.001$, by ANOVA).

(B) Time spent in target zone during the probe test was greater in sham than hippocampal-lesioned mice. Bars represent the time mice spent in the Target zone (that previously housed the platform) during a 60 second test session conducted with the platform removed. As showed in grey bars, sham mice gradually spent more and more time in the target zone, indicating the formation of spatial memory. In contrast, lesioned mice do not show this increase over time ($P < 0.001$, by ANOVA, n=9, 9).

4.2 Hippocampal lesions impaired recent, but not remote, cocaine CPP memory

Contextual fear memories have been shown to reorganize in a time-dependent manner. That is, the hippocampus is necessary for recent, but not remote, contextual fear memories. In this series of experiments, we determined whether a similar pattern is found in cocaine-induced CPP memory. We adapted Kim and Fanselow's experimental design, such that hippocampal lesions were performed either 1 day (recent group: a 1-day old memory being tested) or 30 days (remote group: a 30-day old memory being tested) after CPP conditioning. Tests were conducted 10 days after surgery so that mice had sufficient time to recover, similar to our previously published studies (Wang et al, 2008). The experimental design is depicted as follows:

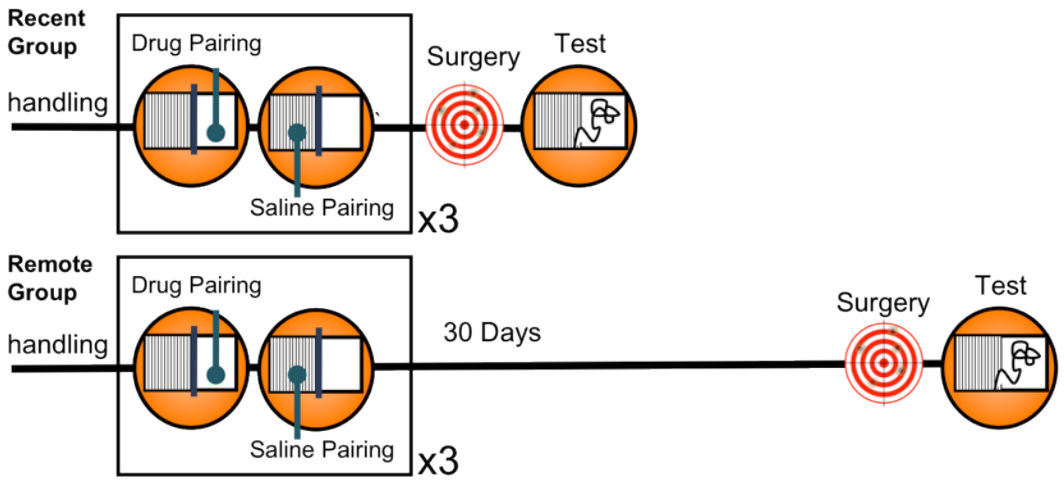


Figure 4.3 Schematic of experimental design to investigate the temporal gradient of hippocampal lesions on cocaine-induced CPP memory.

4.2.1 Recent cocaine CPP was blocked by hippocampal lesions

Experimental procedure

Mice (n=20) were trained as described previously. One day after the 6-day conditioning phase (Day 7), mice were randomly assigned to the lesion or sham-lesion condition. Mice were given 10 days to recover then tested in CPP (see Figure 4.4A).

Test

In the test, we observed that while mice in the sham condition showed a robust preference for the cocaine-paired side, mice in the lesion condition showed no preference (see Figure 4.4B).

An ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Side* (time spent on the cocaine-paired side versus saline-paired side) as a within-subject factor revealed a significant *Surgery* x *Side* interaction [$F(1,18)=23.378$, $P<0.001$], as well as significant main effects of *Side* [*Side* $F(1,18)=65.78$, $P<0.001$; *Surgery* $F(1,18)=2.832$, $P>0.05$; Figure 4.4B]. Post-hoc analyses conducted on the significant interaction (paired *t-test* for the amount of time spent in cocaine-paired versus saline-paired side) showed that sham mice spent more time on the cocaine-paired side than saline-paired side [$t(10)=11.87$, $P<0.001$], but mice with hippocampal lesions spent equivalent time on either side [$t(8)=1.85$, $P>0.05$] (Figure 4.4B). This result indicates that hippocampal lesions conducted 1 day after training impaired subsequent cocaine CPP. CPP data from individual mice is also shown as the heat map in Figure 4.4C. The color of each grid represents the preference score in 5-second intervals during the test. Preference for the saline-paired side is color coded as red and preference for the cocaine-paired side is color coded as blue. The deepness of the color shade indicates the strength of the preference. Collectively, these data indicate that hippocampal lesions conducted one day after training impaired subsequent expression of a recently-acquired cocaine CPP memory. As expected, hippocampal lesioned mice exhibit hyperactive motor behavior, which is a typical hippocampal lesions-induced behavior. Unpaired t-test revealed that mice in the lesion group travelled greater distance compared to the sham mice (unpaired *t*-tests [$t(15)=-3.92$, $P<0.01$]) (Figure 4.4 D). It is unlikely that the difference seen in preference is due to locomotor activity as lesion mice are mobile and active. Moreover, through the following experiments, with additional cohorts of hippocampal

lesion mice, we have also shown that the impairment of cocaine CPP is not due to the difference between locomotion (see discussion).

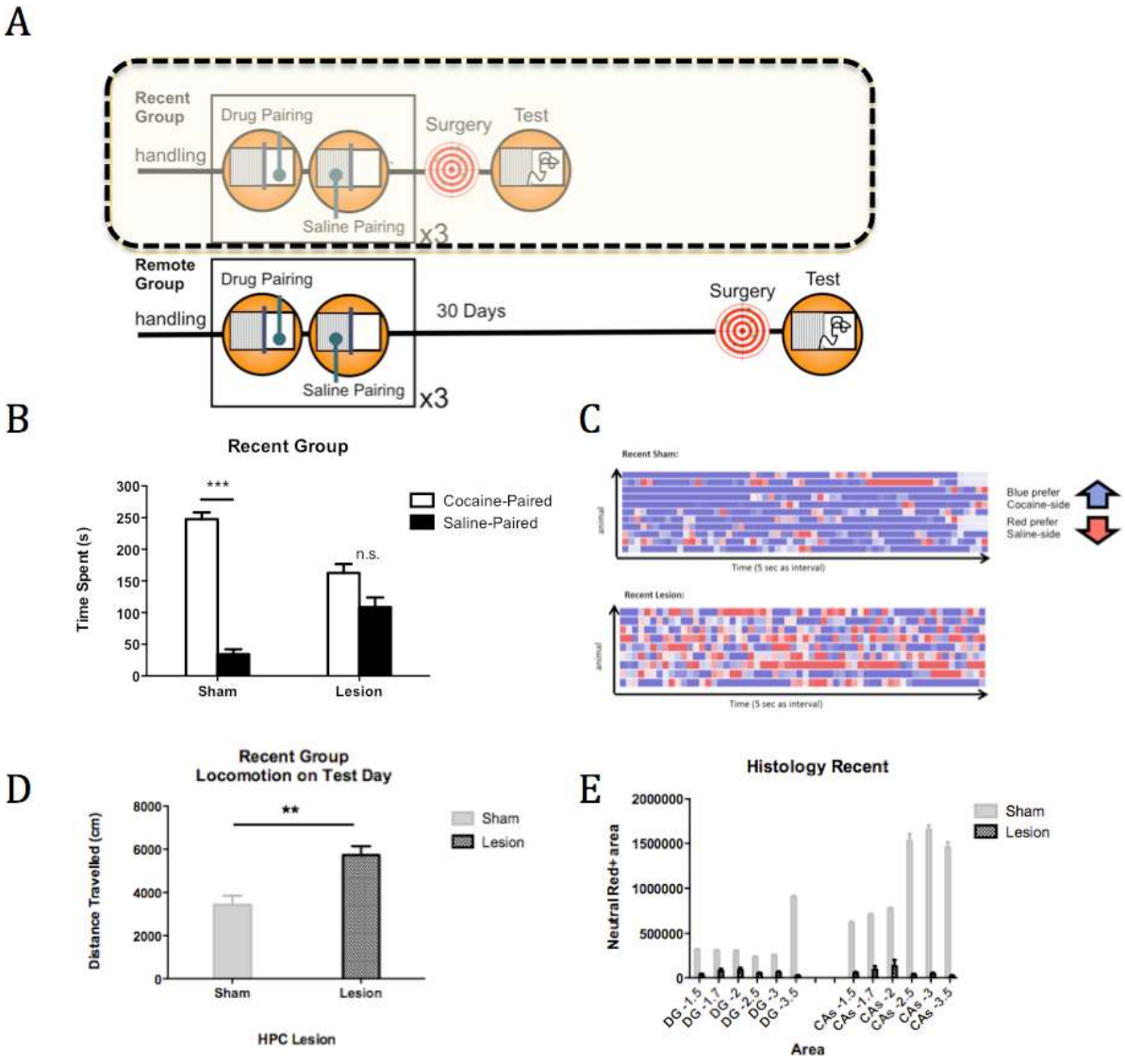


Figure 4.4 Recent cocaine CPP was blocked by hippocampal lesions.

A. Experimental design.

B. Time spent in the cocaine- and saline-paired side during the test session in mice that received sham or hippocampal lesion 1 day following conditioning (recent group). Bars represent the time (seconds) spent in each side. Sham mice showed a significantly higher preference for the cocaine-paired side than mice with hippocampal lesions (P <0.001, by ANOVA, *Surgery* x *Side* interaction), suggesting hippocampal lesions 1 day after training

impaired recent CPP memory.

C. The “Heat map” of individual animals’ preference during the test session. Note that the color of each grid represents the preference score in a 5-second interval. Red: the score for saline-paired side. Blue: the score for cocaine-paired side. The deeper the shade reflects the strength of preference.

D. Locomotion on the test day. As expected, mice with hippocampal lesions travelled more as hyperactivity is typically observed in hippocampal lesioned mice ($P < 0.01$, unpaired *t*-test).

E. Quantification of hippocampal lesions in the recent group. NMDA infusions led to complete (>80%) neuronal depletion in the dorsal and ventral hippocampus ($P < 0.001$, by ANOVA, main effect of Surgery). Data are presented as mean ± SEM. *p< 0.05, **p < 0.01, ***p< 0.001 by paired t-test, n=11 for the sham group and n=9 for the lesion group).

Histology

All histological analyses were performed by another experimenter blind to conditioning subgroup assignment and final test result. Infusion of NMDA induced large, specific hippocampal lesion that resulted in more than 80% cell loss in the dorsal and ventral CA fields and dentate gyrus of the hippocampus, with negligible extra-hippocampal damage. Some small local damage around the needle tracts could be seen in the cortex (somatosensory and parietal) overlying the hippocampus in both sham and lesion groups. Representative lesions are indicated with neutral red staining (Figure 4.5). The extent of hippocampal damage was quantified by measuring the area of cell loss. An ANOVA revealed that cell counts of the hippocampus in lesioned animals were significantly lower than in sham groups [$F(1,11)=836.98$, $P<0.001$] (Figure 4.4E).

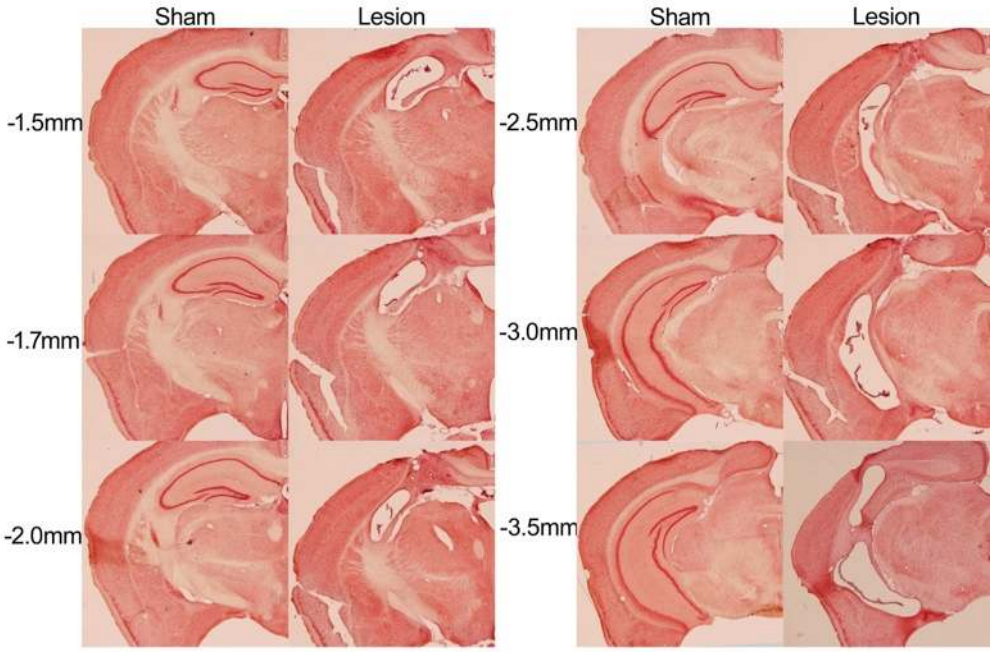


Figure 4.5 Representative hippocampal lesions by neutral red staining.

Representative images of sections from ~1.5 mm to ~3.5 mm posterior to bregma, stained with neutral red. The dorsal and ventral hippocampus was extensively lesioned, with only roughly < 20% of the entire area of the hippocampus remaining.

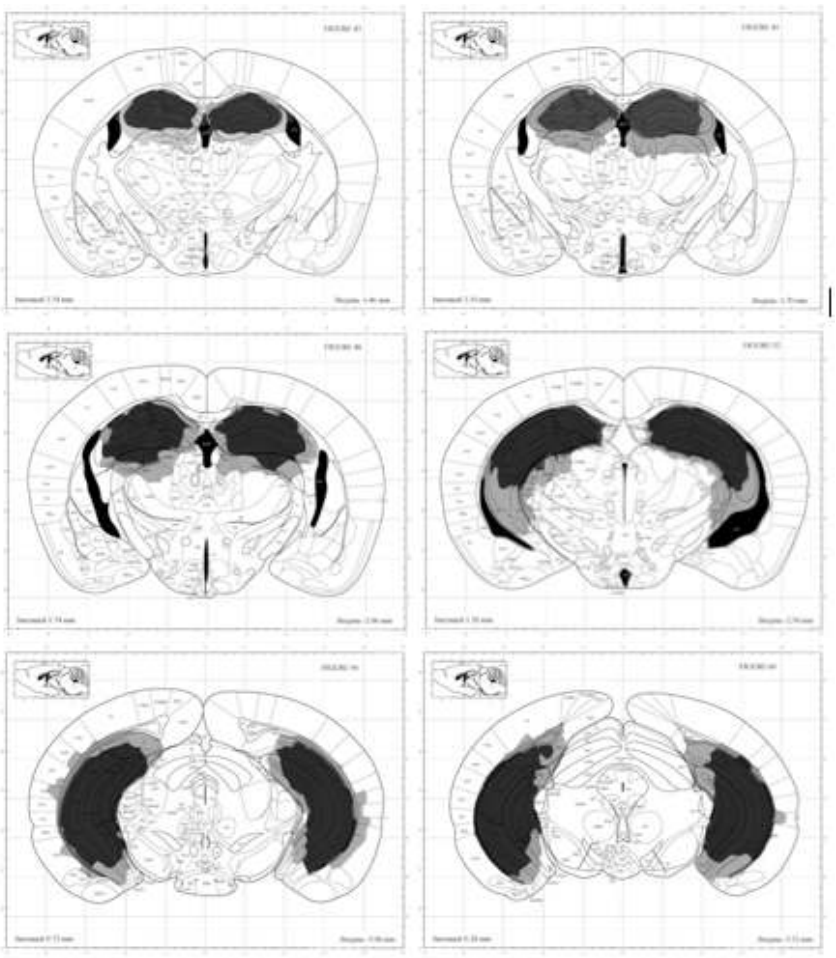


Figure 4.6 Representative diagram of area of hippocampal lesions.

Areas of smallest (black) and largest (grey) lesions are indicated. Modified from the mouse brain atlas of Franklin and Paxinos (Paxions & Franklin, 2001).

4.2.2 Remotely acquired cocaine CPP was not blocked by hippocampal lesions

Previous contextual fear conditioning experiments show that contextual fear memories that initially depend on the hippocampus for their expression may become independent on the hippocampus at more remote time points. We similarly observed in our experiments that recent cocaine CPP memory was sensitive to hippocampal lesions. To test whether remote cocaine CPP memory requires the hippocampus, we waited 30 days after training and then performed hippocampal lesion surgeries.

Experimental procedure

Mice (n=21) were trained with the cocaine CPP protocol as described previously and, 30 days after the last session of conditioning were randomly assigned into lesion or sham conditions. Hippocampal or sham lesion surgeries were performed as above. Mice were given 10 days to recover (see Figure 4.7) and tested for cocaine CPP.

Test

During the test, we observed that both groups of mice (sham and lesion conditions) showed a robust preference for the cocaine-paired side. There was no significant difference in preference for the cocaine-paired side between lesion and sham group. An ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Side* (cocaine-paired side versus saline-paired side) as a within-subject factor was conducted on the data. There was no significant *Surgery* x *Side* interaction [$F(1,19)=0.31$, $P>0.05$] or main effect of *Surgery* $F(1,19)=0.10$, $P>0.05$] but a significant effect of *Side:* $F(1,19)=32.00$, $P<0.001$], indicating that both groups showed cocaine CPP. These data suggest that the remote cocaine CPP memory do not depend on hippocampus.

Time spent in cocaine-paired versus saline-paired sides was compared using paired *t*-tests in both groups. These analyses revealed that both sham mice [$t(11)=4.33$, $P<0.01$] and lesion mice [$t(8)=3.97$, $P<0.01$] spent more time on the cocaine-paired side versus saline-paired side, indicating a cocaine CPP.

Collectively, these data suggest that hippocampal lesions 30 days after training did not impair cocaine CPP. A similar pattern was also observed in the heat map of preference (Figure 4.7). As expected, hyperactive behavior was again observed in mice with hippocampal lesion (unpaired *t*-tests [t (19)=-2.77, P <0.05]) (Figure 4.7D). This excludes the possibility that hippocampal lesions non-specifically impaired the cocaine CPP previously observed, as the preference could still be demonstrated in mice with typical hyperactive behavior induced by hippocampal lesions.

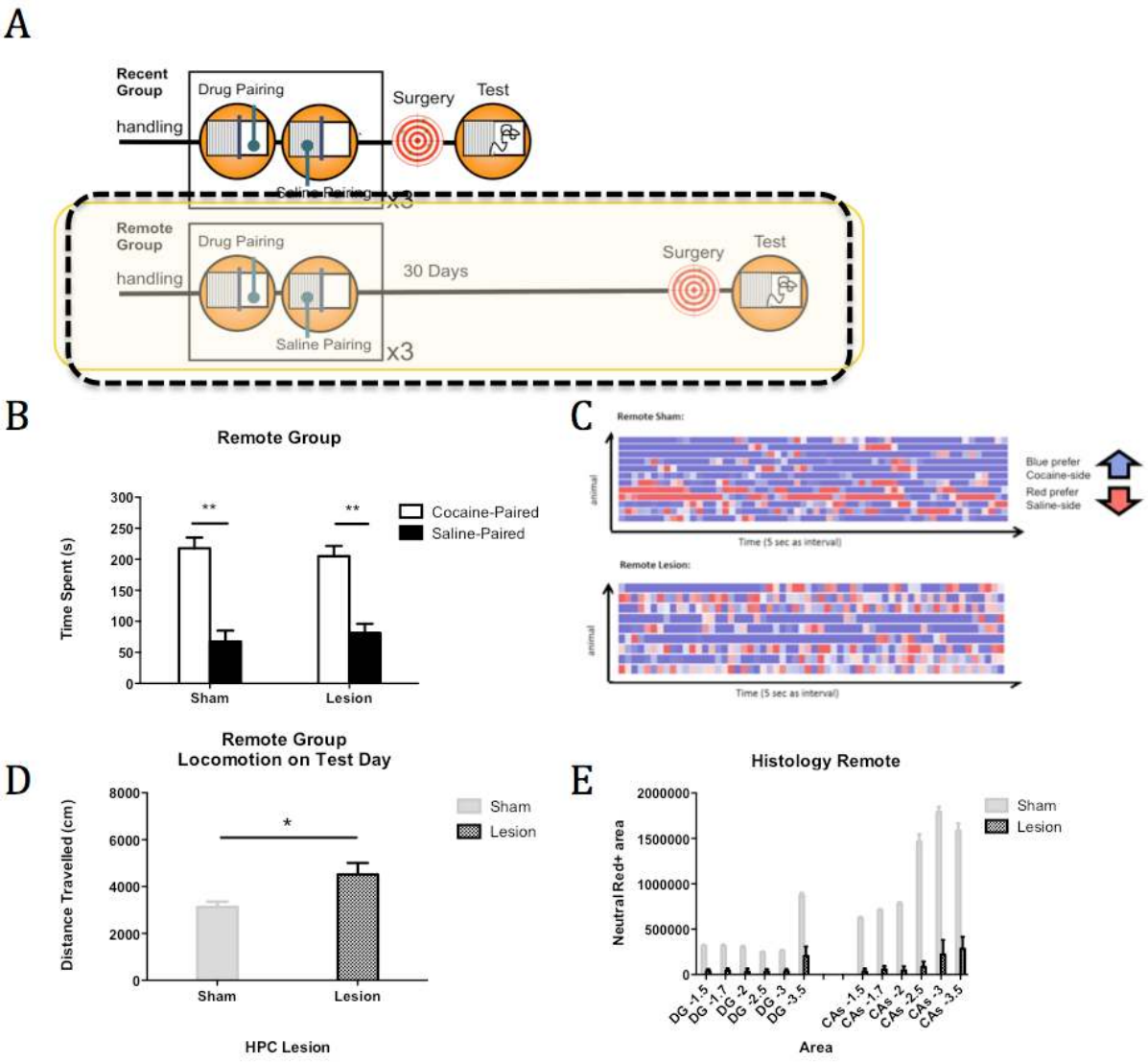


Figure 4.7 Remotely-acquired cocaine CPP was not blocked by hippocampal lesions.

A. Experimental design.

B. Time spent in the cocaine- versus saline-paired side during the test session in sham and lesion mice (remote group; surgeries performed 30 days after training). Bars represent the time (seconds) spent in each side. Both groups of mice showed preference for the cocaine-paired compartment, suggesting hippocampal lesions at this time point did not impair remote cocaine CPP memory.

C. The "Heat map" of individual animals' preference during the test session. Note that the color of each grid represents the preference score in a 5-second interval. Red: the score for saline-paired side. Blue: the score for cocaine-paired side. The deeper the shade reflects the strength of preference.

D. Locomotion on test day. As expected, mice with hippocampal lesions travelled more than sham-lesioned mice (P <0.01, by unpaired t-test).

E. Quantification of hippocampal lesions in the remote group. NMDA infusions produced complete (>80%) neuronal depletion in the dorsal and ventral hippocampus (P <0.001, by ANOVA, main effect of Surgery). Data are presented as mean ± SEM. *p< 0.05, **p < 0.01, ***p< 0.001 by paired t-test, n=12 for the sham operated group and n=9 for the hippocampal lesion group.

F. Locomotion on the test day. As expected, mice with hippocampal lesions travelled more than non-lesioned mice (P <0.01, unpaired t-test).

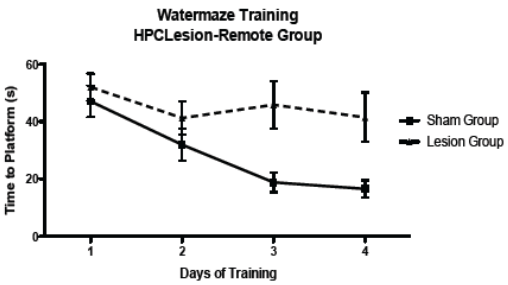
Water maze

We observed no effect of hippocampal lesion on remote cocaine CPP memory. To verify the effectiveness of the hippocampal lesions in these mice, we trained and tested these mice in the water maze. In our previous experiments (see Figure 4.8) and published paper (Wang et al., 2009). We showed that the hippocampal lesion impaired the formation of a spatial water maze memory in otherwise experimentally naïve mice. We used the same protocol here. Specifically, mice were trained in water maze for 4 consecutive days, with 2 training trials per block and 2 blocks per day. The probe test was conducted on day 4. During the probe test, the hidden platform was removed and the mice were allowed to explore freely for 60 seconds. Mice swim paths were automatically tracked and recorded.

We observed that during training, mice in the lesion group showed significant impairment in learning the location of the submerged platform. The latency to reach the hidden platform was higher in the lesion group. An ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Time* (4 days) as a within-subject factor revealed main effects of *Surgery* and *Time* [*Surgery:* $F(1,19)=8.92$, $P<0.001$; *Time:* $F(1,7)=6.01$, $P<0.001$; *Surgery* x *Time:* $F(1,7)=1.90$, $P>0.05$], suggesting a trend that lesioned mice, in general, took more time to reach the platform than non-lesioned mice (Figure 4.8).

In the probe tests, we quantified the amount of time mice spent searching in the target zone (20cm radius circular zone centered on the former platform location) versus the average time spent in the equivalent zones in the other three quadrants of the pool. We observed that sham mice spent more time in the target zone compared to the lesioned mice. An ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Zone* (target versus other) as a within-subject factor revealed a significant interaction between *Surgery* x *Zone* [$F(1, 19)=7.20$, $P<0.05$], indicating that sham operated mice spent more time in the target zone than lesioned mice. Collectively, these data suggest that our NMDA infusions effectively lesioned the hippocampus (as spatial memory acquisition was severely impaired) yet had no effect on remote cocaine CPP (Figure 4.8).

A



B

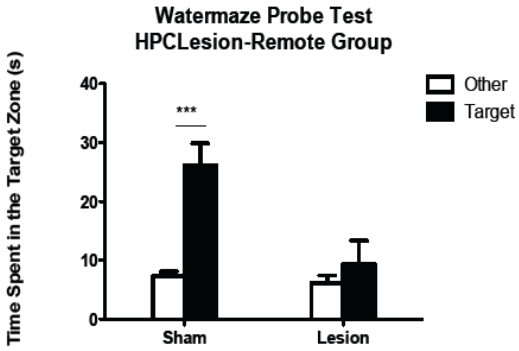


Figure 4.8 Verifying that our NMDA infusions effectively lesioned the hippocampus using lack of spatial memory acquisition in the water maze (remote group)

A. Latency (seconds) to reach the hidden platform during training of the water maze. Over training, lesioned mice took more time to reach the platform, indicating deficits in learning the location of the submerged platform (P <0.001, by ANOVA, main effect of *Surgery*).

B. Time spent in the target zone during the probe test. Lesioned mice spent less time in the target zone (P <0.001, by ANOVA, *Surgery* x *Zone* interaction), indicating deficits in the acquisition of a spatial memory. These data indicate that our infusion in these mice produced effective hippocampal lesions. Data are presented as mean ± SEM. *p< 0.05, **p < 0.01, ***p< 0.001 by paired t-test. N=12 for the sham operated group and n=9 for the hippocampal lesion group.

Histology

As previously described in Materials and Methods, infusions of NMDA produced complete lesions of the hippocampus. The extent of damage to the hippocampus was quantified by measuring the area of remained cells. An ANOVA revealed that cell counts of the hippocampus in lesioned animals were significantly lower than in sham the group [$F(1,17)=186.59$, $P<0.001$] (Figure 4.7E).

4.2.3 Temporal gradient of retrograde amnesia in CPP

To examine CPP across time, we used a CPP difference score, an index of CPP used across many studies (Cunningham et al., 2003). The difference score is calculated by the difference between the raw time spent in cocaine-paired and saline-paired sides, and it directly reflects the preference of the subjects.

We compared the CPP difference score at the recent (1 day) and remote (30 days) time points in mice with hippocampal lesions versus sham-operated controls (Figure 4.9B). A two-way ANOVA with *Surgery* (lesion versus sham) and *Delay* (recent versus remote) as between-subject factors was conducted on the data. Importantly, this analysis revealed that there was a significant interaction between *Surgery* x *Delay* [$F(1,37)=5.01$, $P<0.05$], indicating that the effect of hippocampal lesion on CPP differed between recent and remote time points. There was also a main effect of *Surgery* [$F(1,37)=9.89$, $P<0.01$], indicating that mice with hippocampal lesions in general had lower difference score when compared to the sham mice, while there was no main effect of *Delay* [$F(1,37)=0.02$, $P>0.05$], indicating the total difference scores of mice were equivalent at recent versus remote time points. *Post-hoc* Bonferroni test further revealed that the difference score of sham versus lesion mice at the recent time point was significantly different. Collectively, these data support the idea that circuits supporting drug-associated memories undergo reorganization in a time-dependent manner, such that recent cocaine CPP memory retrieval is hippocampus dependent whereas remote cocaine CPP memory retrieval may not be dependent on the hippocampus.

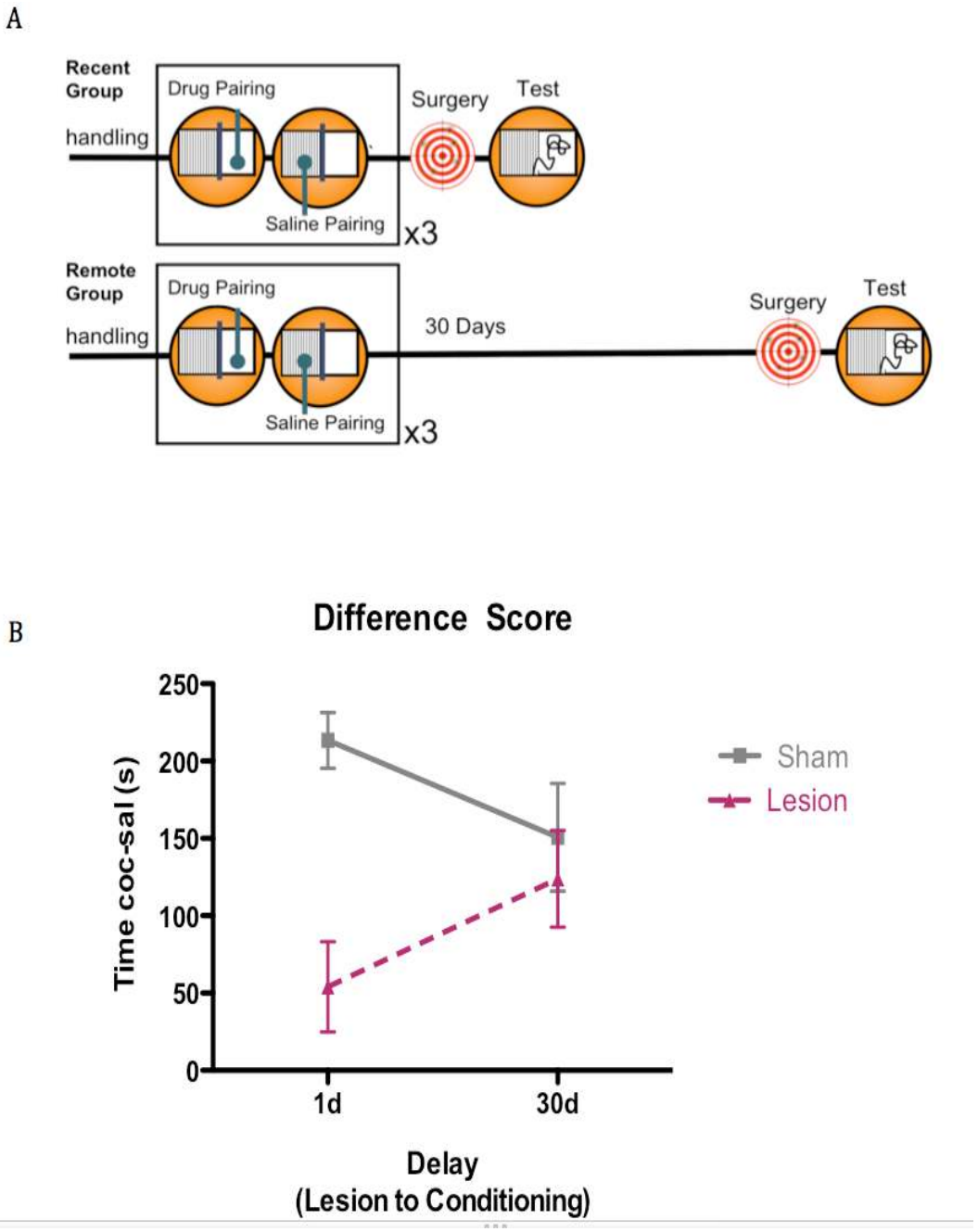


Figure 4.9 The temporal gradient of retrograde amnesia of cocaine CPP.

A. Experimental design to test if the recent (1 day) versus remote (30-day) cocaine CPP memory depended on the hippocampus.

B. CPP difference score compared between the recent and remote groups. CPP difference score is the difference between the time spent on the cocaine-paired side minus time spent on the saline-paired side and reflects the strength of CPP of each subject. Two-way

ANOVA with *Surgery* (lesion versus sham) and *Delay* (recent versus remote) revealed a significant interaction between *Surgery* x *Delay* [$F(1,37)=5.01$, $P<0.05$].

4.3 Nucleus accumbens lesions impaired both recent and remote cocaine-CPP memory

The nucleus accumbens is a brain region implicated in mediating the rewarding properties of drugs of abuse, including cocaine, amphetamine, opiates, nicotine, and alcohol (Wise & Bozarth, 1987; Sora et al., 2001; Self & Nestler, 1995). We have previously showed that cocaine CPP memory was initially hippocampal dependent, but that over time, this memory became hippocampal independent. Next, we asked if there was a similar temporal gradient with manipulations of other brain regions. Previous studies have shown that nucleus accumbens is involved in learning cocaine CPP (Sellings et al, 2006), but little is known about the potential temporal effects of the nucleus accumbens in cocaine CPP memory.

To examine the role of nucleus accumbens in the expression of cocaine CPP at different time points, we adapted the same experimental framework from our hippocampal studies. The experimental design is depicted as follows:

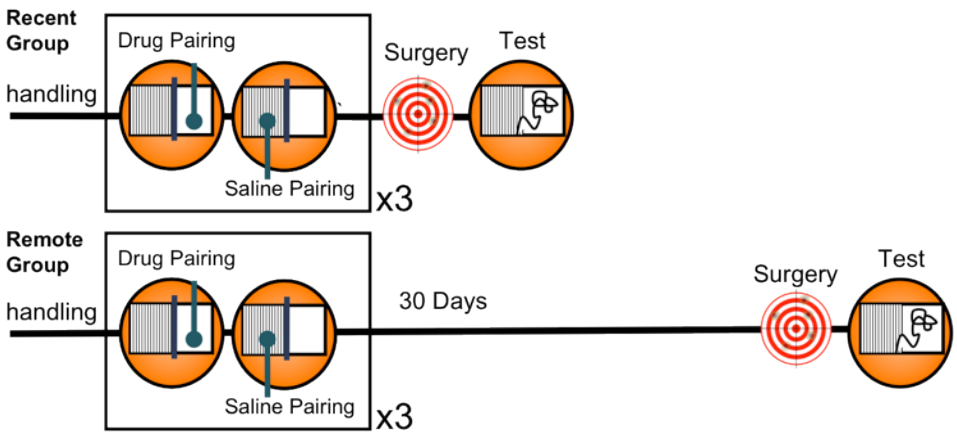


Figure 4.10 Experimental design to examine the role of the nucleus accumbens in reorganization of cocaine CPP memory

4.3.1 Nucleus accumbens lesions impaired recent cocaine CPP expression

Experimental procedure

The experimental design of the nucleus accumbens group is identical with the recent/ remote hippocampus study. Cocaine CPP lasts for 6 days. 1 day after conditioning, mice were randomly assigned to either the lesion or sham conditions. Nucleus accumbens lesion (or sham) surgeries were performed and mice were given 10 days to recover in their homecages.

Test

During the test, we observed that while mice in the sham treatment group showed a robust preference for the cocaine-paired side, mice in the nucleus accumbens lesion group showed no preference. An ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Side* (Cocaine-paired side versus Saline-paired side) as a within-subject factor revealed a *Surgery* x *Side* interaction [$F(1,18)=44.75$, $P<0.001$], indicating that the CPP significantly differed in the lesion versus sham group. There was a main effect of *Side* [*Side:* $F(1,18)=34.06$, $P<0.001$; *Surgery:* $F(1,18)=0.212$, $P>0.05$], indicating that in general mice spent more time on the cocaine-paired side during testing. Post-hoc analyses conducted on the significant interaction (paired t-test for the amount of time spent in cocaine-paired versus saline-paired side) showed that sham mice spent more time on the cocaine-paired side versus saline-paired side [$t(9)=9.51$, $P<0.001$], but mice with nucleus accumbens lesions spent equivalent time on either side [$t(9)=0.58$, $P>0.05$] (Figure 4.11B). Collectively, these data indicate that nucleus accumbens lesions one day after training impaired expression of cocaine CPP. Moreover, no significant effects on the distance travelled between sham and lesion group was found by using unpaired *t*-tests [$t(18)=-0.75$, $P>0.05$] (Figure 4.11C).

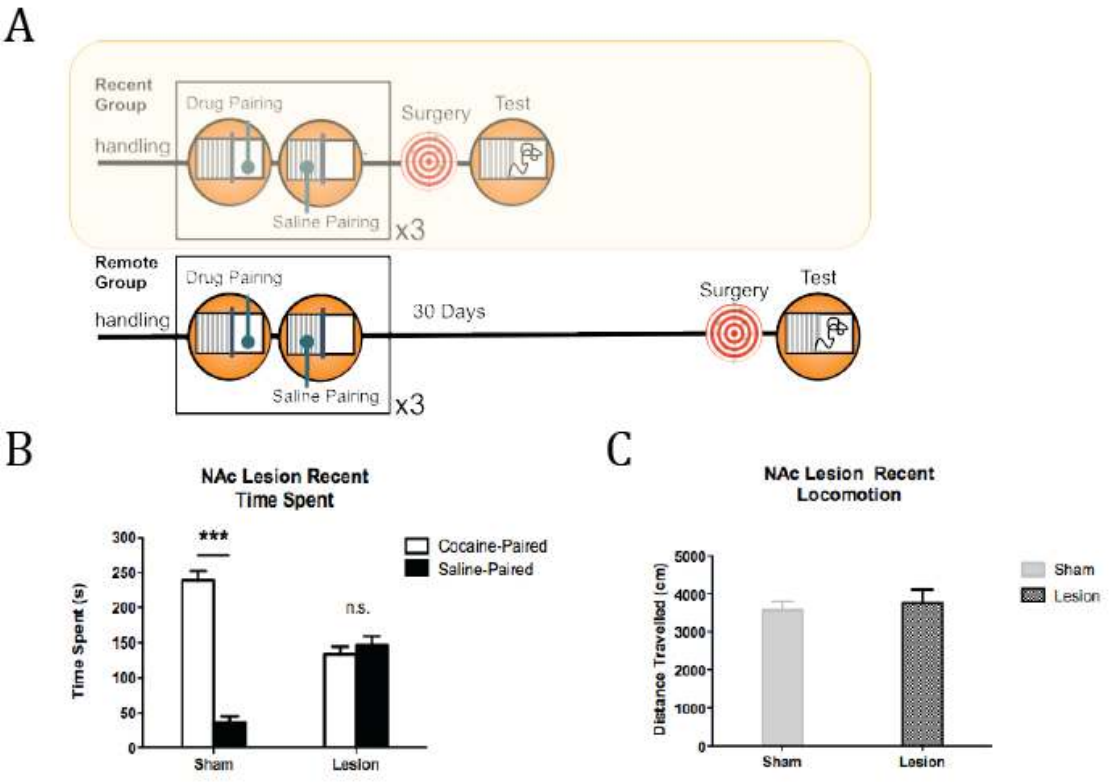


Figure 4.11 Cocaine CPP was blocked by nucleus accumbens lesions performed 1 day after training.

A. Experimental design.

B. Time spent in the cocaine- versus saline-paired side during the test session of mice that received nucleus accumbens (NAc) lesion or sham treatment. Bars represent the time (seconds) spent in each side. The preference of sham mice for the cocaine-paired side was significantly higher than mice with nucleus accumbens lesion (P <0.001, by ANOVA, *Surgery* x *Side* interaction), suggesting that nucleus accumbens lesions impaired recent cocaine CPP.

C. Locomotion activity on the test day. Mice with nucleus accumbens lesions travelled similar distance compared to the sham lesioned mice during the test. Data are presented as mean ± SEM. *p< 0.05, **p < 0.01, ***p< 0.001 by paired t-test.

Histology

All histological analyses were performed by another experimenter who was blind to conditioning subgroup assignment and final test result. Histological analysis showed accumbens lesion resulted in cell loss or cell disorganization restricted to nucleus accumbens (core and shell), with minimum damage to other brain regions (Figure 4.12 and Figure 4.13). Mice in the lesioned group were included if >80% of the lesion was located in the nucleus accumbens region. Neuronal loss extended in an antero-posterior direction from approximately 2.0 mm to 0.6 mm anterior to bregma, and did not extend ventrally or caudally into the ventral pallidum or olfactory tubercle. Some small local damage around the needle tracts could be observed in the cortex overlying the nucleus accumbens in both sham and lesion groups.

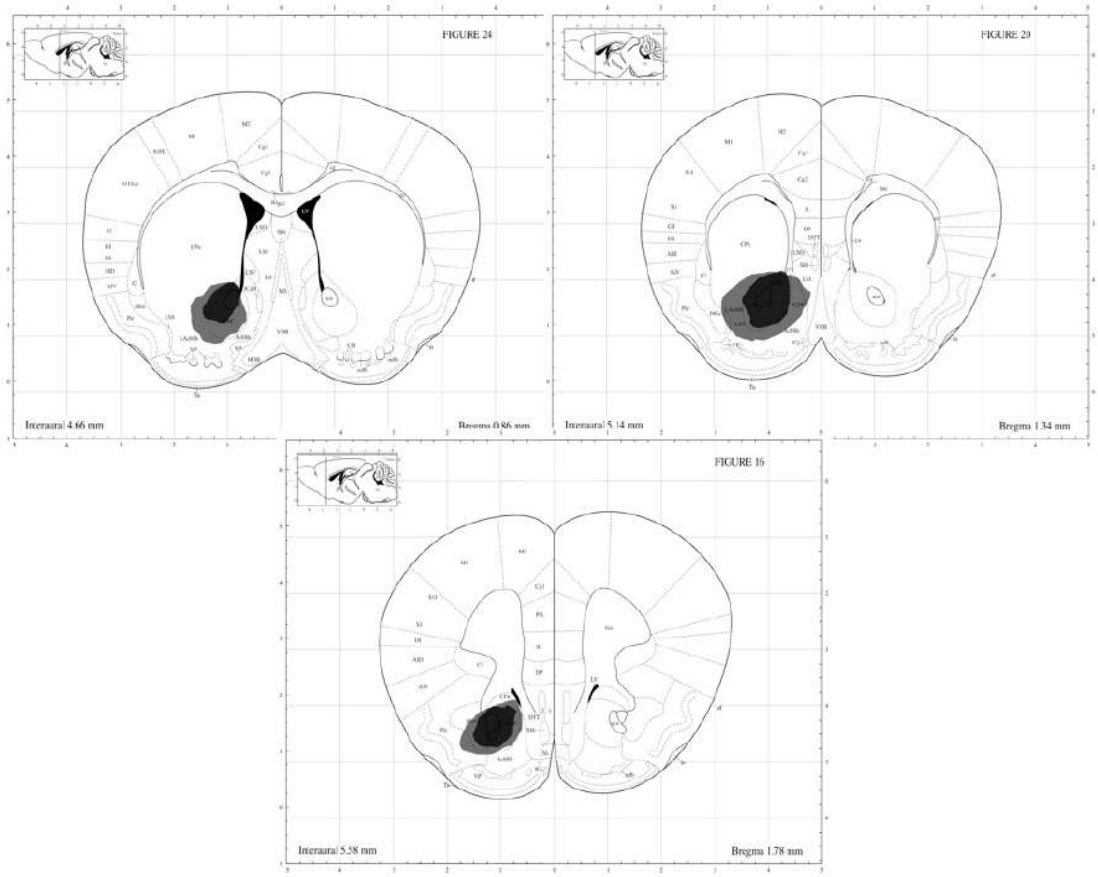


Figure 4.12 Representative diagram of the nucleus accumbens lesions.

Areas of the smallest (black) and largest (grey) lesions are indicated. Modified from the mouse brain atlas of Franklin and Paxinos (Paxions and Franklin, 2001) .

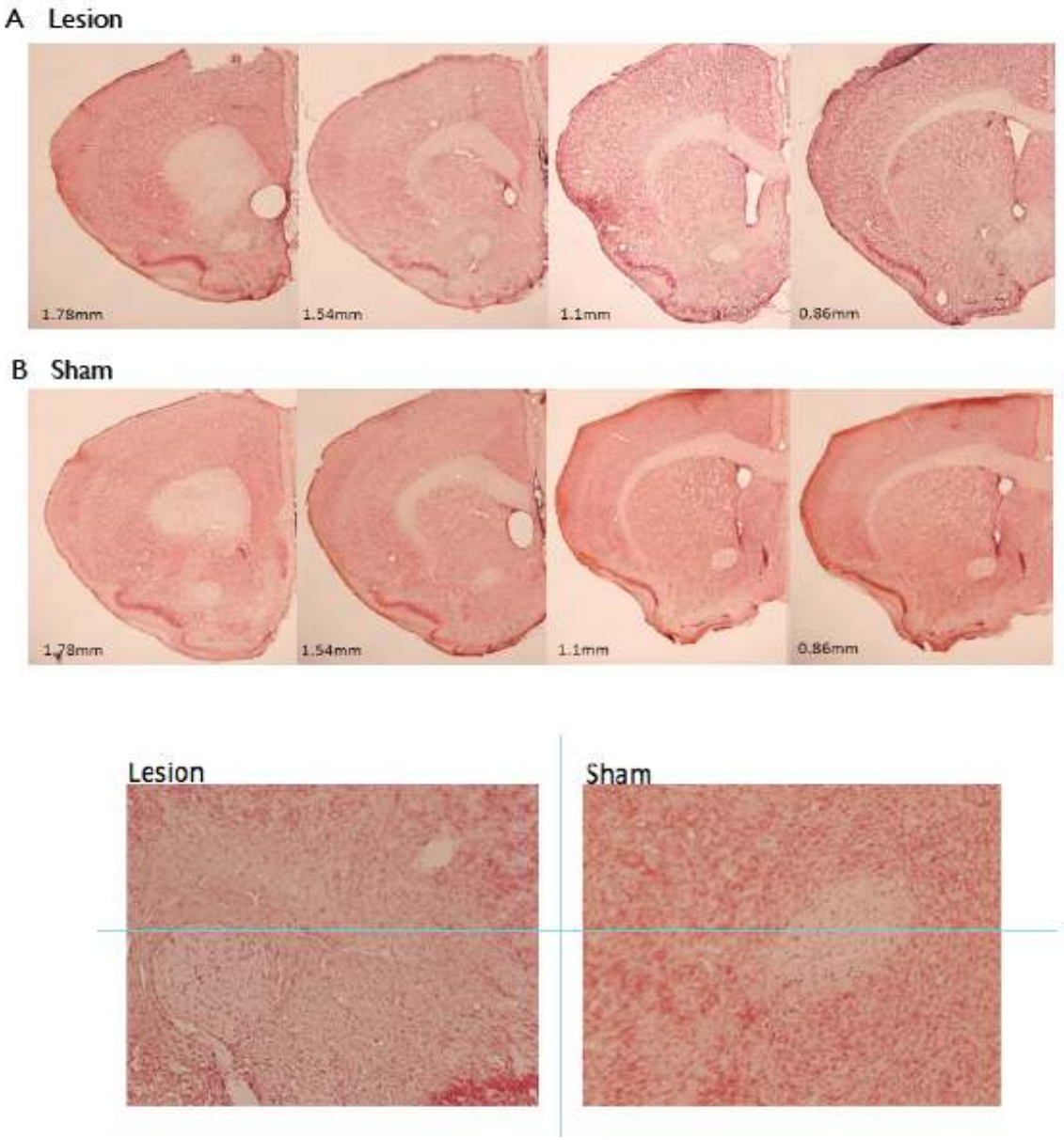


Figure 4.13 Representative pictures of lesions in the nucleus accumbens.

Brain sections from 1.78 - 0.86 mm anterior to bregma, stained with neutral red.

(A) Lesion-operated mice, low-magnification view, left hemisphere (medial to the right)

(B) Sham-operated mice, low-magnification view, left hemisphere (medial to the right)

(C) High-magnification view on lesion- and sham- operated mice. Note in the region of the lesion, neurons have been replaced by disorganized and loosely-stained cells, indicating cell loss.

4.3.2 Nucleus accumbens lesions impaired remote cocaine CPP expression

Experimental procedure

Experimental procedures were as previously described. Naïve mice were trained in cocaine CPP protocol for 6 days. 30 days after conditioning, mice were randomly assigned to nucleus accumbens lesion or sham condition. Mice were given 10 days to recover post-surgery.

Test

During the test, we observed that the preference for the cocaine-paired side significantly differed between nucleus accumbens lesion and sham group, as the sham, but not lesion, mice exhibited a strong preference for the cocaine-paired side. An ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Side* (cocaine-paired side versus saline-paired side) as a within-subject factor revealed a *Surgery* x *Side* interaction [$F(1, 26)=5.52$, $P<0.05$] (Figure 4.14B), indicating that the place preference differed in the lesion versus sham group. There was a main effect of *Side* [*Side:* $F(1, 26)=18.46$, $P<0.001$; *Surgery:* $F(1,26)=0.24$, $P>0.05$], indicating that mice in general spent more time on the cocaine-paired side during testing. To assess these differences more directly, time spent in cocaine-paired versus saline-paired sides was compared using paired *t*-tests in both groups. These analyses revealed that whereas sham mice spent more time in the cocaine-paired side versus saline-paired side [$t(15)=5.71$, $P<0.001$], mice with hippocampal lesions spent equivalent time on either side [$t(9)=0.56$, $P>0.05$]. Collectively, these data indicate that nucleus accumbens lesions 30 days after training impaired expression of a remote cocaine CPP memory. No significant effects on the distance travelled between sham and lesion group was found using unpaired *t*-tests [$t(24)=-0.16$, $P>0.05$] (Figure 4.14C).

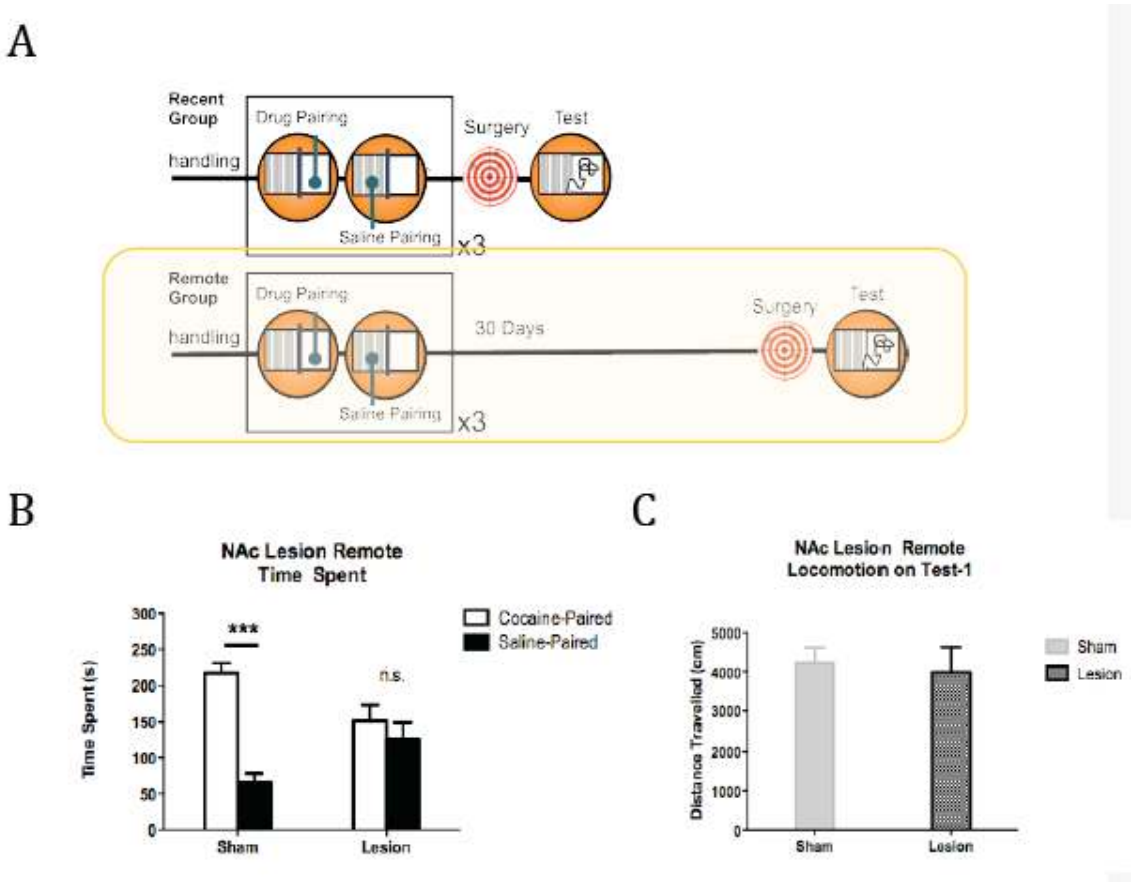


Figure 4.14 Remote cocaine-induced CPP was blocked by nucleus accumbens lesions.

A. Experimental design.

B. Time spent in the cocaine- versus saline-paired side during the test session of mice with sham and nucleus accumbens (NAc) lesion performed 30 days following conditioning. Bars represent the time (seconds) spent in each side. The preference of sham mice for the cocaine-paired side was significantly higher than the nucleus accumbens lesion mice (P <0.001, by ANOVA, *Surgery* x *Side* interaction), suggesting that nucleus accumbens lesions significantly impaired remote cocaine CPP.

C. Locomotion activity during the test. Mice with nucleus accumbens lesions travelled similar distance compared to the sham lesioned mice during the test. Data are presented as mean ± SEM. *p< 0.05, **p < 0.01, ***p< 0.001 by paired t-test.

4.3.3 Comparing the effects of Nucleus accumbens lesions on the recent versus remote cocaine CPP

The combined data from recent and remote nucleus accumbens lesion groups suggest that nucleus accumbens lesions disrupt both recent (1-day old) and remote (30-day old) cocaine CPP memory. The analysis of CPP difference scores supports this conclusion. A two-way ANOVA with *Surgery* (lesion versus sham) and *Delay* (recent versus remote) as between-subject factors was conducted on the CPP difference scores. Importantly, there was no *Surgery* x *Delay* interaction [$F(1, 44)=3.33$, $P>0.05$], indicating that nucleus accumbens lesions disrupted CPP both at recent and remote time points (Figure 4.15). There was a main effect of *Surgery* [$F(1, 44)=28.79$, $P<0.001$], indicating that mice with nucleus accumbens lesions exhibited significantly lower difference scores compared to the sham mice, independent of *Delay* [$F(1, 44)=0.92$, $P>0.05$]. Collectively, these data support the idea that nucleus accumbens plays a critical role in the expression of both recent and remote cocaine CPP.

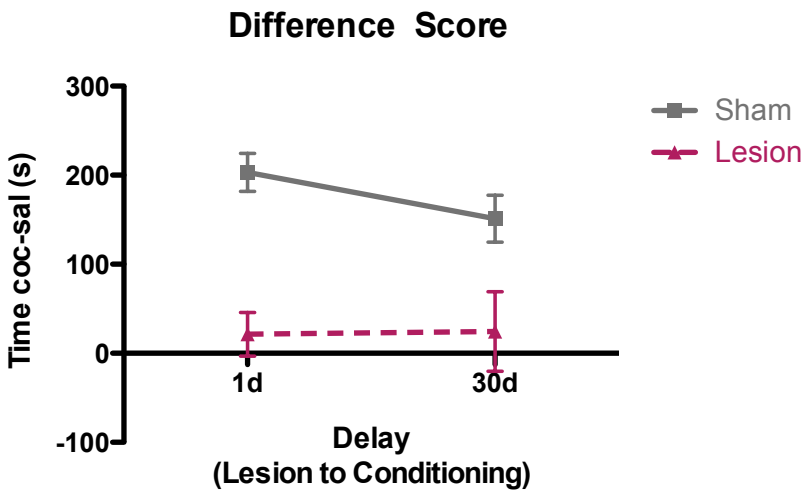


Figure 4.15 Comparing the effects of nucleus accumbens lesions on recent versus remote cocaine CPP.

Nucleus accumbens lesions blocked both recent and remote cocaine CPP, indicating that, unlike hippocampus, the nucleus accumbens is involved in both recent and remote cocaine CPP.

4.4 Reengagement of hippocampus in remote cocaine CPP

Systems reconsolidation has been demonstrated by Debiec and colleagues, who reported that exposure to a reminder rendered a remote (hippocampal independent) memory into a hippocampal *dependent* state (Debiec et al., 2002). Interestingly, two major theories of systems consolidation posit different roles of the hippocampus in this systems reconsolidation. In contrast to standard consolidation theory, multiple trace theory predicts that only the context-free memory would reorganize, whereas the context-specific memory would remain hippocampus-dependent, even at remote time points (Winocur et al., 2007). Therefore, when reminders reactivate a remote memory, the memories regain the context specificity, thus becoming hippocampal-dependent again. Indeed, it has been demonstrated in the discriminative contextual fear paradigm that remote memories gradually lose context specificity. Moreover, only a context-specific (not context-general) reminder can bring a remote memory back to being hippocampal dependent (Winocur et al., 2009).

Based on reminder studies, we hypothesized that remote (hippocampal-independent) cocaine CPP memory would become re-sensitive to hippocampal damage after reactivation by exposure to reminders. To test this in the remote cocaine CPP, we gave different reminders (drug+ paired context, context only, drug alone) of varying strength one day before the hippocampal lesion. As in our previous experiments, mice were allowed to recover for 10 days after surgery. In this experiment, the no-reminder group was a complete replica of our earlier remote group experiment, as no reminder was given to mice. In the drug+context (strong) reminder case, mice were injected with cocaine (12 mg/kg i.p.) before being confined in the cocaine-paired context for 5 min one day before surgery. In the context only (weak) reminder, mice were confined to the cocaine-paired context for 5 min (drug-free). In the drug only (weak) reminder, mice were injected with cocaine (12mg/kg i.p.) in their homecages.

The experimental design is depicted as follows:

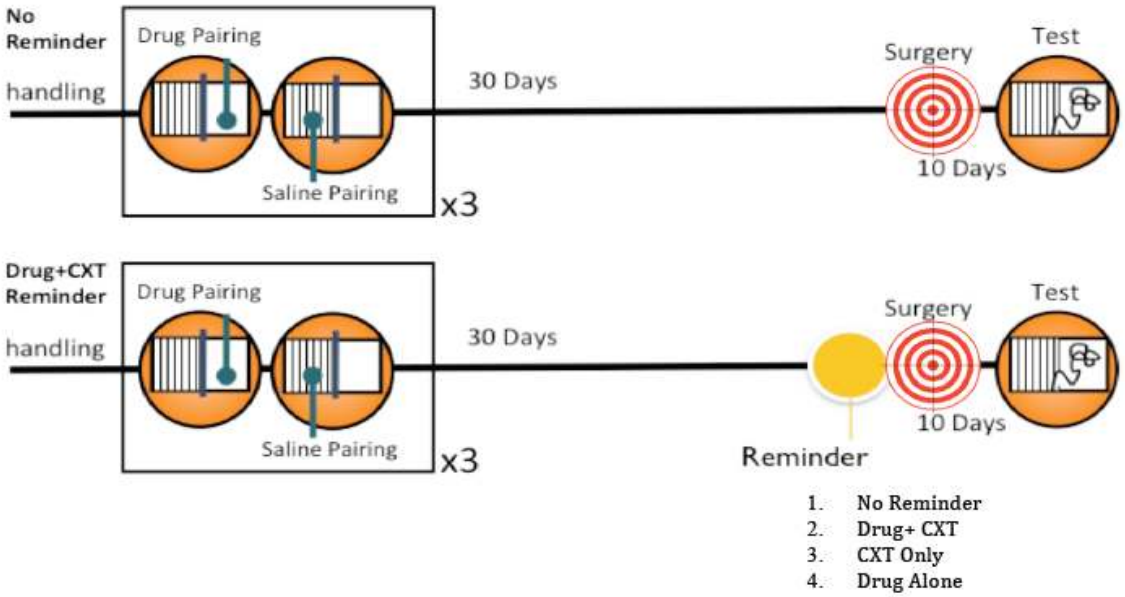


Figure 4.16 Experimental design to investigate re-engagement of hippocampus in remote cocaine CPP memory.

4.4.1 No reminder group

Experimental procedure

Training protocol is identical to the recent/ remote experiment and has been previously described. In the no-reminder group, no reminder was given before surgery. Thirty days after conditioning, mice were randomly assigned into lesion or sham groups and hippocampal lesion surgeries were performed. Mice were given 10 days to recover.

Test

In the no-reminder group, we observed remote hippocampal lesion did not affect cocaine CPP (see Figure 4.16). An ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Side* (cocaine-paired side versus saline-paired side) as a within-subject factor revealed no *Surgery* x *Side* interaction [$F(1,15)=0.62$, $P>0.05$], indicating that the place preference did not differ in the lesion versus sham group. However, the main effect of *Side* [*Side:* $F(1,15)=28.64$, $P<0.001$ and *Surgery:* $F(1,15)=1.36$. $P>0.05$], indicated that in general mice spent more time on the cocaine-paired side during testing, regardless of surgery. Time spent in cocaine-paired versus saline-paired sides was compared using paired *t*-tests in both groups. Paired t-tests revealed that both sham mice [$t(8)=3.91$, $P<0.01$] and lesioned mice [$t(7)=3.89$, $P<0.01$] spent more time on the cocaine-paired side versus saline-paired side. Again, these data indicate that hippocampal lesions did not impair expression of a remotely acquired (30-day old) cocaine CPP. In this group, hippocampal lesioned mice showed similar levels of hyperactivity with lesioned mice in other groups, however sham mice in this group also showed increased levels of locomotor behaviour, possibly due to the increased anxiety from changes in housing. Therefore no significant effects on the distance travelled between sham and lesion group was found in this group [unpaired *t*-tests, $t(15)=-1.07$, $P>0.05$] (Figure 4.17). Subsequent learning of the water maze task verified the behavioral effects of the hippocampal lesions in these mice.

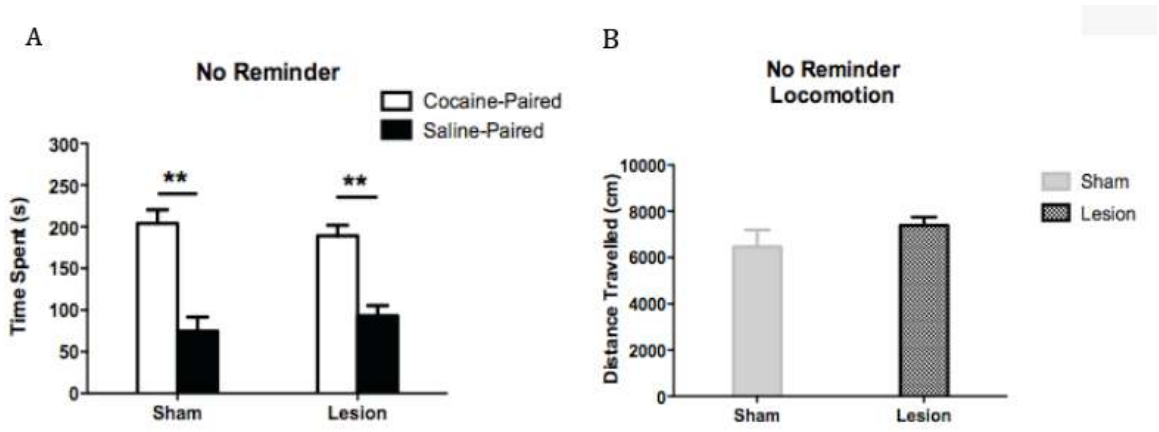


Figure 4.17 Reengagement of the hippocampus in remote cocaine CPP: No-reminder group.

A. Time spent in the cocaine- and saline-paired side during the test session in sham and lesioned mice (No reminder group). Bars represent the time (seconds) spent in each side. Both sham and lesion mice showed significant CPP for the cocaine-paired side, suggesting that hippocampal lesions after no reminder did not block remote cocaine CPP.

B. Locomotion on test day. In this experiment, high levels of locomotor activity was found in both lesioned and sham mice. While the lesion mice showed typical level of increased locomotion similar to our other hippocampal groups, sham mice also showed increased level of locomotion possibly due to housing changes. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by paired t-test.

Water maze

To further verify the effectiveness of the hippocampal lesions in these mice, mice were subsequently trained in water maze task.

During training sessions, lesioned mice showed a slower learning curve reflected by the increase in latency to reach hidden platform compared to sham-treated mice. An ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Time* (4 days) as a within-subject factor revealed a main effect of *Surgery* and of *Time* [*Surgery: F*(1,15)=7.38, *P* <0.05; *Time: F*(1,15)=10.50, *P* <0.001], indicating that lesion mice in general spent more time to reach the platform (Figure 4.18A).

In the probe tests, lesioned mice also showed deficit in spatial memory. Sham mice spent more time in the target zone compared to lesioned mice. An ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Zone* (target versus other) as a within-subject factor showed a *Surgery* x *Zone* interaction [*F*(1, 15)=5.20, *P* <0.05], indicating that time spent in target zone differed between lesion and sham groups. A main effect of *Zone* and *Surgery* [*Zone: F*(1,15)=14.48, *P* <0.01; *Surgery: F*(1,15)=8.50, *P*<0.01], indicated that mice in general spent more time in target zone versus other zones. Collectively, these data again verify that our infusions produced hippocampal lesions in these mice (Figure 4.18).

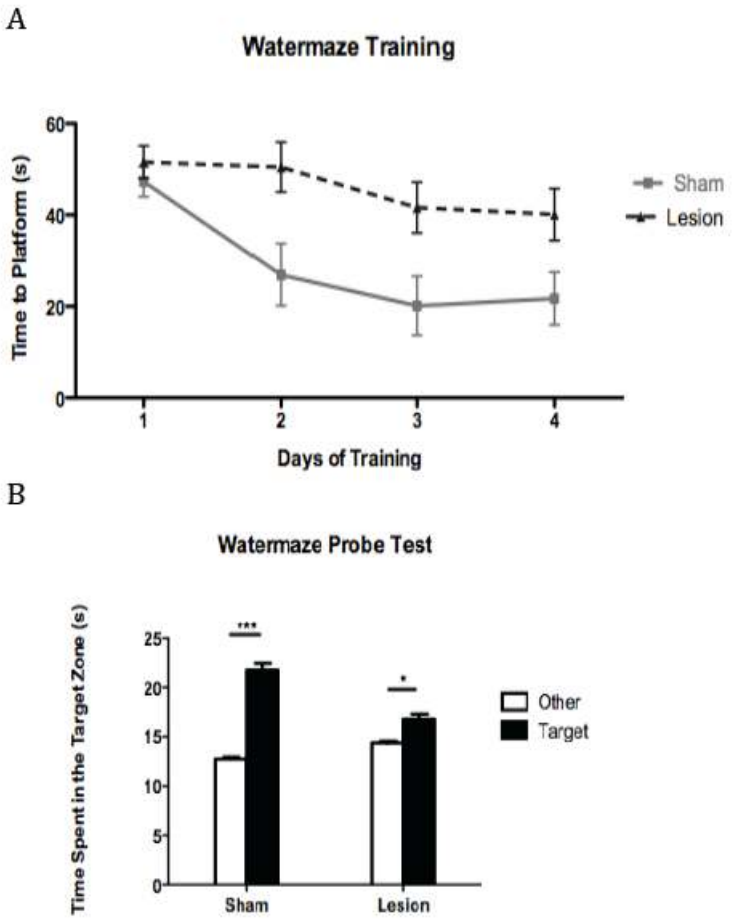


Figure 4.18 Verifying the hippocampal lesion in no-reminder CPP experiment by using the water maze.

A. Latency to the hidden platform during training. Line represents the time spent to reach the hidden platform on over training. As shown in the solid line, lesioned mice in general spent more time to reach the platform, indicating deficits in learning the location of the platform (P <0.001, by ANOVA, main effect of *Surgery*).

B. Time spent in the target zone during the test. Bars represent the time mice spent in the target versus other zones (in a 60-second test session). Again, sham mice spent significantly more time in the target zone (P <0.001, by ANOVA, *Surgery* x *Zone* interaction) compared to the lesioned mice. These data indicate that hippocampal lesions were effective in impairing the acquisition of a spatial memory (in the water maze) and verify the lack of effect on remote CPP. Data are presented as mean ± SEM. *p< 0.05, **p < 0.01, ***p< 0.001 by paired t-test.

Histology

As previously described, our infusion produced large lesions of the hippocampus. The extent of damage to the hippocampus was quantified by measuring the area of cell loss. A main effect of ANOVA revealed that cell counts of the hippocampus in lesioned animals were significantly lower than in sham groups in [$F(1,11)=646.90$, $P<0.001$].

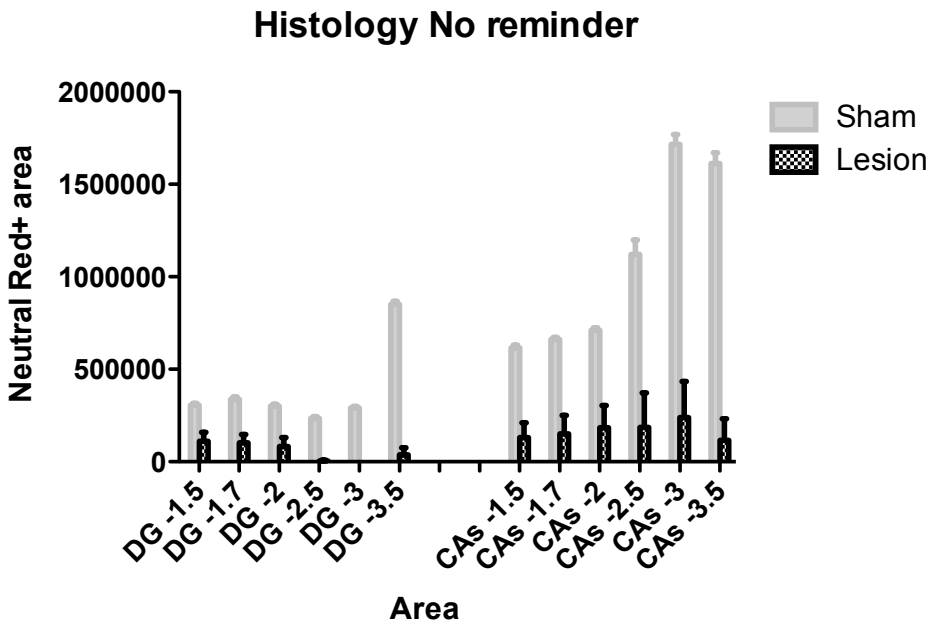


Figure 4.19 Quantification of hippocampal lesions in no-reminder remote CPP group.

NMDA lesions produced near complete neuronal depletion in the dorsal and ventral hippocampus in the no-reminder group ($P<0.001$, by ANOVA, main effect of *Surgery*).

4.4.2 Drug plus context reminder

Experimental procedure

Training procedures were described previously. In the drug+ context group, mice were injected with cocaine (12 mg/kg i.p.) and then confined to the cocaine-paired context for 5 min one day before surgery. Hippocampal lesion surgeries were performed as previously described. The delay between conditioning to surgery remained 30 days. Mice were given 10 days to recover after surgery.

Test

Hippocampal lesions impaired expression of a remotely-acquired cocaine CPP if mice were given a drug+context reminder the day before hippocampal lesion. Sham-treated mice, as expected, showed a strong preference for the cocaine-paired compartment. An ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Side* (cocaine-paired side versus saline-paired side) as a within-subject factor revealed a *Surgery* x *Side* interaction [$F(1, 25)=15.19$, $P<0.001$; *Side:* $F(1, 25)=26.95$, $P<0.001$; *Surgery:* $F(1,25)=0.13$, $P>0.05$], indicating that the place preference differed in the lesioned versus sham groups. Importantly, Time spent in cocaine-paired versus saline-paired compartments was compared using paired *t*-tests in both groups. Paired *t*-test analyses revealed that sham mice spent more time on the cocaine-paired side versus saline-paired side [$t(15)=6.34$, $P<0.001$], whereas mice with hippocampal lesions spent equivalent time on either side [$t(10)=1.08$, $P>0.05$]. Collectively, these data indicate that hippocampal lesions 1 day after a strong reminder (drug+context reminder) impaired expression of a 30 day-old cocaine CPP. As expected, mice with hippocampal lesion showed high levels of activity during the test (unpaired *t*-tests [$t(25)=-5.57$, $P<0.001$], Figure 4.20B).

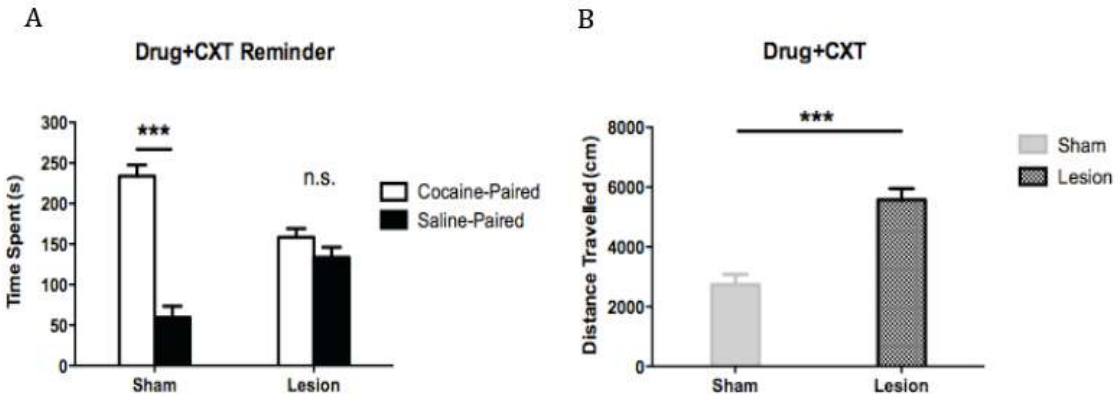


Figure 4.20 Reengagement of hippocampus in remote CPP: drug+context reminder.

A. Time mice spent during the CPP test in the cocaine- versus saline-paired compartment following a strong reminder (drug+context experiment) and subsequent lesion of the hippocampus. The preference of sham mice for the cocaine-paired compartment was significantly higher than saline-paired compartment, while mice with hippocampal lesions spent equal time in cocaine- and saline-paired compartments, suggesting hippocampal lesions impaired remote CPP memory after drug+context reminder ($P < 0.001$, by ANOVA, *Surgery* x *Side* interaction).

B. Locomotion on test day. Mice with hippocampal lesions travelled more during the test than sham operated control mice. Data are presented as mean ± SEM. *p< 0.05, **p < 0.01, ***p< 0.001 by paired t-test.

Histology

As previously described, the hippocampus was entirely lesioned. The extent of damage to the hippocampus was quantified by measuring the area of cell loss. A main effect of ANOVA revealed that cell counts of the hippocampus in lesioned animals were significantly lower than in sham groups [$F(1, 17)=1212.09$, $P<0.001$].

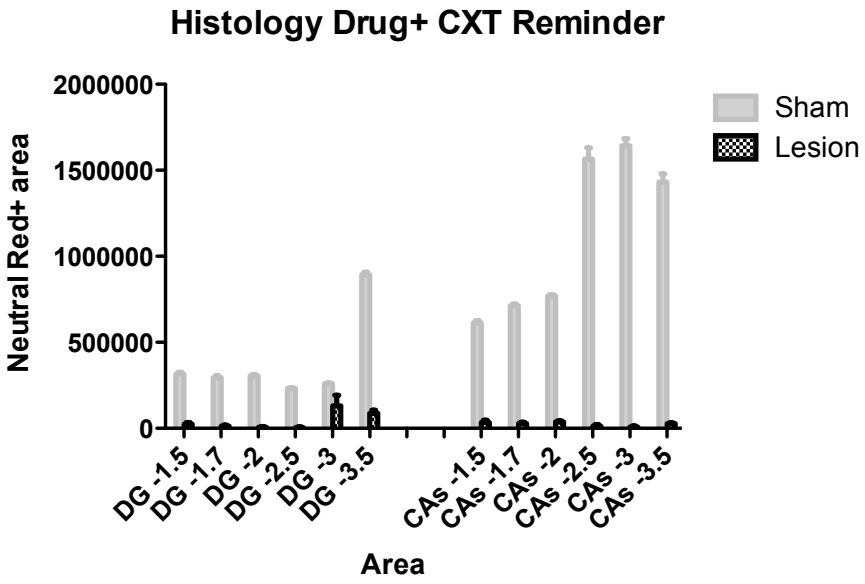


Figure 4.21 Quantification of hippocampal lesions in the drug+context reminder experiment.

NMDA lesions led to almost complete neuronal depletion in the dorsal and ventral hippocampus in drug+context reminder group ($P<0.001$, by ANOVA, main effect of *Surgery*).

4.4.3 Comparing no-reminder with drug+context reminder groups

The data from no-reminder and drug+context reminder groups suggest that hippocampal lesions after a strong reminder disrupted a remote cocaine-associated contextual memory (Figure 4.22). A two-way ANOVA with *Surgery* (lesion versus sham) and *Group* (no-reminder versus drug+context reminder) as between-subject factors showed a *Surgery* x *Group* interaction [$F(1,40)=8.25$, $P<0.01$], indicating that the effect of hippocampal lesions on CPP differed between no-reminder and drug+context reminder groups. *Post-hoc* Bonferroni test further revealed that only the difference score of sham versus lesion mice in drug+context reminder group was significantly different. There was a main effect of *Surgery* [$F(1,40)=9.2$, $P<0.01$], indicating that mice with hippocampal lesions in general exhibited lower difference score when compared to the sham mice, while there was no main effect of *Group* [$F(1,40)=0.04$, $P>0.05$], indicating the total difference scores of mice were equivalent between no-reminder and drug+context reminder groups. Collectively, these data support the idea that following a strong reminder, a remote hippocampus-independent CPP memory becomes hippocampal-dependent.

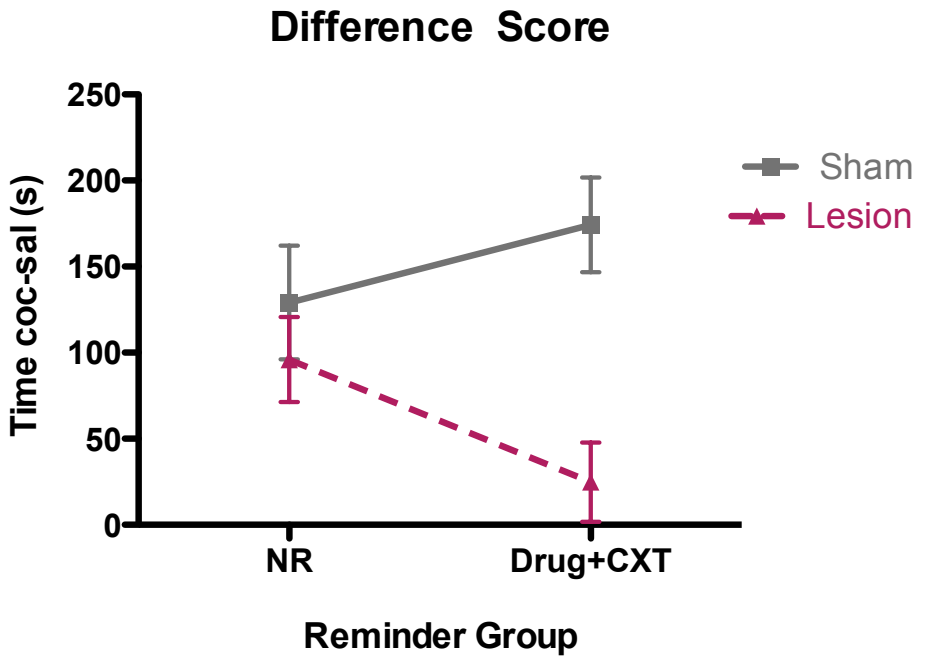


Figure 4.22 Reengagement of hippocampus in remote cocaine CPP memory.

Comparing the effect of hippocampal lesions on difference scores between no-reminder (NR) and drug+context (Drug+CXT) reminder experiments (P <0.01, by ANOVA, *Surgery* x *Group* interaction). In the drug+context reminder group, sham mice had significantly higher difference score than lesioned mice, suggesting that with a strong reminder, remote hippocampus-independent cocaine CPP memory became susceptible to hippocampus lesions again.

4.4.4 Weak reminders

4.4.4.1 Drug alone reminder

Experimental procedure

Mice were trained as described previously. In the drug alone reminder, mice were injected with cocaine (12 mg/kg i.p.) in their homecages. Hippocampal lesion surgeries were performed as previously described. The delay between conditioning to surgery remained 30 days. Mice were given 10 days to recover after surgery.

Test

We observed that reminder of a remotely acquired hippocampal-independent CPP with exposure to cocaine alone facilitates re-engagement of the hippocampus. In this drug-alone reminder experiment, we observed that the sham mice showed a greater preference compared to the lesioned mice (see Figure 4.23). An ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Side* (cocaine-paired side versus saline-paired side) as a within-subject factor revealed a *Surgery* x *Side* interaction [$F(1, 13)=5.05$, $P<0.05$], indicating that the preference differed in the lesion versus sham group. There was a main effect of *Side* [*Side:* $F(1, 13)=112.47$, $P<0.001$; *Surgery:* $F(1,13)=0.00$, $P>0.05$], indicating that mice in general spent more time on the cocaine-paired side during testing. Time spent in cocaine-paired versus saline-paired sides was compared using paired *t*-tests in both groups. Paired *t*-tests revealed that both sham mice [$t(7)=15.29$, $P<0.001$] and mice with hippocampal lesions [$t(6)=4.36$, $P<0.01$] spent more time on the cocaine-paired side versus saline-paired side, indicating that both groups of mice showed preference for the cocaine-paired side. Collectively, these data suggest that a weak reminder partially re-engaged the hippocampus. Hippocampal lesions impaired, but did not completely abolish, the preference for the drug-context. As expected, hippocampal lesion produced high levels of activity during the test (unpaired *t*-tests, $t(13)=-3.56$, $P<0.01$).

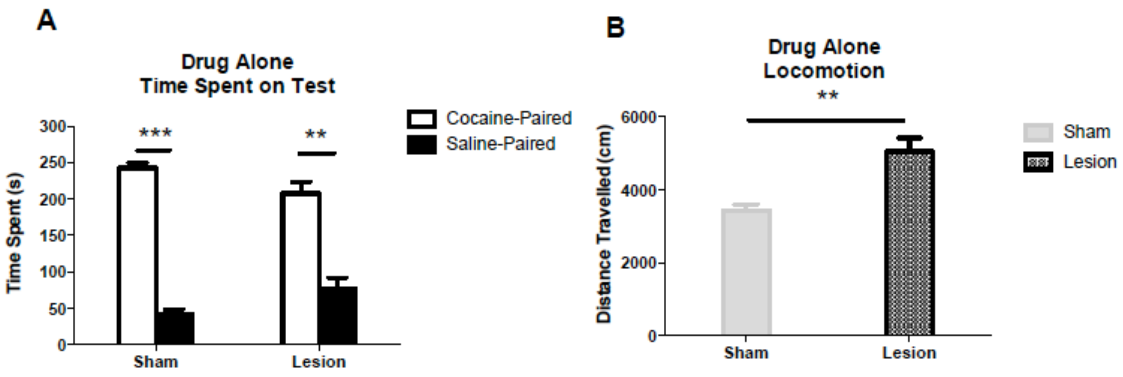


Figure 4.23 Re-engagement of hippocampus in remote CPP: drug-alone reminder.

A. Time spent in the cocaine- versus saline-paired compartments during the test session (drug-alone reminder experiment). Bars represent the time mice spent in each side. Both sham and lesion mice exhibited significant CPP for the cocaine-paired side; however, the preference is weaker in lesioned mice (P <0.05, by ANOVA, *Surgery* x *Side* interaction), suggesting a drug-alone reminder re-engages the hippocampus.

B. Locomotion on test day. Mice with hippocampal lesions travelled more than sham-treated mice. Data are presented as mean ± SEM. *p< 0.05, **p < 0.01, ***p< 0.001 by paired t-test.

Histology

As previously described, the hippocampus was entirely lesioned. A main effect of ANOVA revealed that cell counts of the hippocampus in lesioned animals were significantly lower than in sham groups in all areas [$F(1, 10)=1428.51$, $P<0.001$].

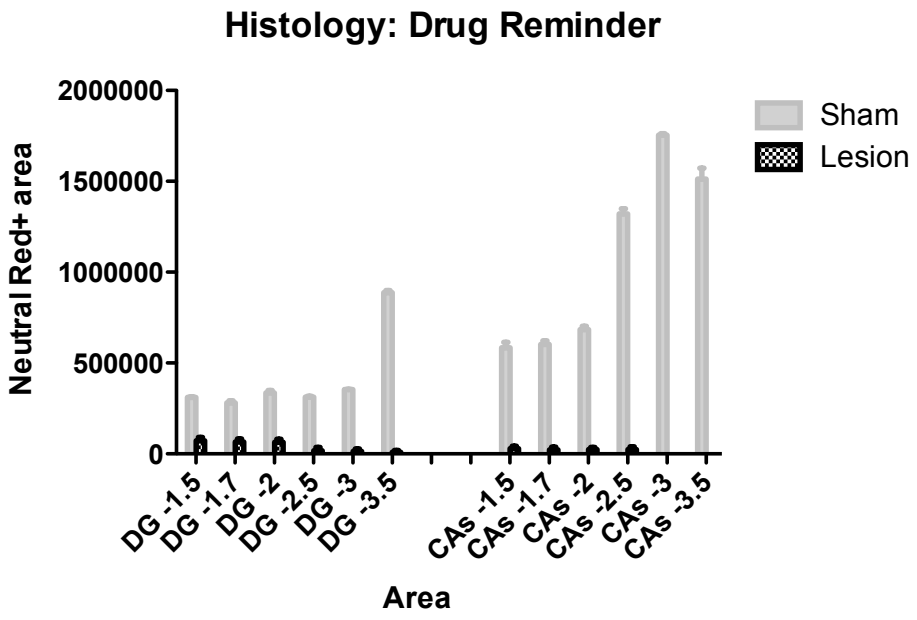


Figure 4.24 Quantification of hippocampal lesions in drug-alone reminder experiment.

NMDA lesions produced almost complete neuronal depletion in the dorsal and ventral hippocampus in drug-alone reminder experiment ($P<0.001$, by ANOVA, main effect of *Surgery*).

4.4.4.2 Context only reminder

Experimental procedure

Mice were trained as described in previous section. In the context-only reminder group, mice were placed in the cocaine-paired context for 5 minutes (drug-free) the day before hippocampal lesion surgery. Hippocampal lesion surgeries were performed as previously described. The delay between conditioning to surgery remained 30 days. Mice were given 10 days to recover after surgery.

Test

Hippocampal lesions impaired expression of a remotely acquired cocaine CPP if a context-only reminder was performed the day before lesion. An ANOVA with *Surgery* (lesion versus sham) as a between-subject factor and *Side* (cocaine-paired side versus saline-paired side) as a within-subject factor revealed a *Surgery* x *Side* interaction [$F(1, 25)=4.78$, $P<0.05$], indicating that the place preference differed in the lesioned versus sham groups. Main effect of *Side* [*Side:* $F(1, 25)=38.83$, $P<0.001$; *Surgery:* $F(1.25)=1.07$, $P>0.05$] indicated that mice in general spent more time on the cocaine-paired side during testing. To assess these differences directly, time spent in cocaine-paired versus saline-paired sides was compared using paired *t*-tests in both groups. These analyses revealed that both sham mice [$t(16)=6.52$, $P<0.001$] and mice with hippocampal lesions [$t(10)=2.89$, $P<0.01$] spent more time on the cocaine-paired side versus saline-paired side, indicating both groups of mice exhibited the preference for the cocaine-paired context. Collectively, these data indicate that a context-only reminder was sufficient to re-engage the hippocampus in remotely-acquired cocaine CPP. As expected, hippocampal lesion was also associated with high activity during the test (unpaired *t*-tests [$t(25)=-4.28$, $P<0.001$] (Figure 4.25).

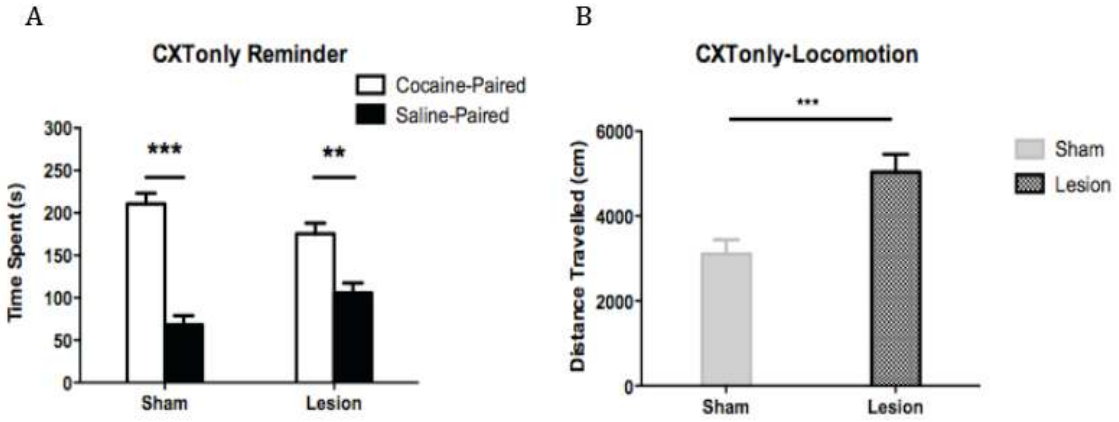


Figure 4.25 Re-engagement of hippocampus in remote CPP: context-only reminder experiment.

A. Time spent in the cocaine- versus saline-paired compartments on the test day in context-only reminder experiment. Both sham and lesion mice showed CPP for the cocaine-paired side; however, the preference was greater in the sham-treated mice (P <0.05, by ANOVA, *Surgery* x *Side* interaction), suggesting a context-only reminder re-engages the hippocampus.

B. Locomotion on test day. Mice with hippocampal lesions travelled more than sham-treated mice. Data are presented as mean ± SEM. *p< 0.05, **p < 0.01, ***p< 0.001 by paired t-test.

Histology

As previously described, the hippocampus was entirely lesioned. A main effect of ANOVA revealed that cell counts of the hippocampus in lesioned animals were significantly lower than in sham groups in all areas [$F(1, 17)=890.63$, $P<0.001$].

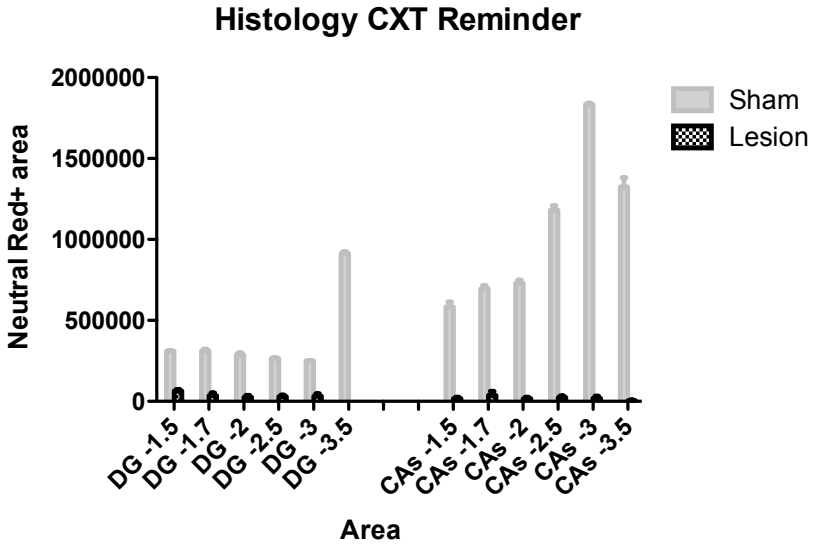


Figure 4.26 Quantification of hippocampal lesions in context-only experiment.

NMDA lesions led to almost complete neuronal depletion in the dorsal and ventral hippocampus in context only reminder group ($P<0.001$, by ANOVA, main effect of *Surgery*).

4.4.5 Comparing all reminder experiments

Here, we showed that remote cocaine CPP memories re-engage the hippocampus if "revived" by different reminders (see Figure 4.27). These data suggest that complete re-engagement of hippocampus required a reminder in which the drug was again paired with the context (producing a faithful re-enactment of a training trial), as hippocampal lesion blocked a 30-day old cocaine CPP in the strongest reminder group (drug+context). However, in the weak reminder groups (drug -alone or context-only reminders), hippocampal lesions produced a mild impairment of remote cocaine CPP. With significant statistical differences between sham and lesion mice, the preference for cocaine-paired context could still be observed (Figure 4.27).

A similar pattern of results was shown in the contextual fear-conditioning paradigm. A stronger context-specific reminder, but not a weak context-general reminder, effectively re-engaged the hippocampus in an otherwise remote hippocampal-independent contextual fear memory (Winocur et al., 2009).

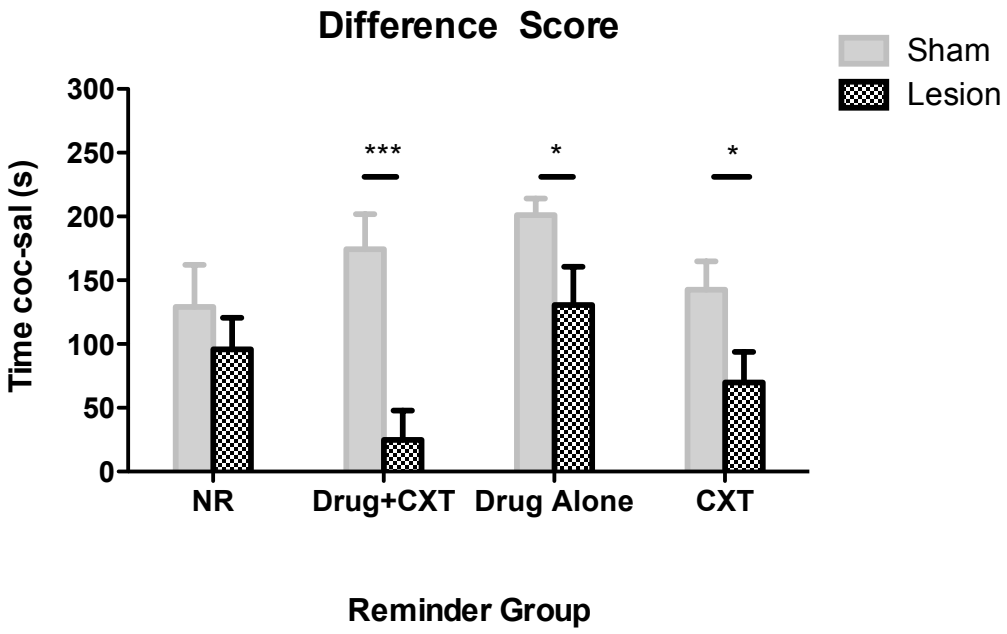


Figure 4.27 Reconsolidation of CPP memory: comparing all reminder experiments.

Hippocampal lesions completely blocked expression of a remote (hippocampal-independent) CPP if the lesions were performed 1 day following a strong reminder (drug+context). In contrast, memory was more modestly impaired if lesions were performed 1 day following weak reminders (drug-alone and context-alone). Data are presented as mean ± SEM. *p< 0.05, **p < 0.01, ***p< 0.001 by paired t-test.

4.5 MEF2 and CPP

Myocyte Enhancer Factor 2 (MEF2) is a transcription factor that negatively regulates spine growth in an activity dependent manner both *in vitro* and *in vivo*. We have previously showed that locally and acutely increasing MEF2 function in hippocampus impaired spatial memory formation (Vetere et al, 2011; Cole et al, 2012). In this critical study by Cole et al (2012), it was further demonstrated that decreasing MEF2 function in hippocampus and amygdala facilitated the formation of spatial and fear memory, respectively. The authors demonstrated MEF2-rgulated spine growth is critical in the formation of fear conditioning and spatial water maze memory. These bidirectional effects suggest that MEF2 inhibits the structural plasticity necessary for memory and therefore inhibits memory formation.

To investigate whether this structural plasticity in the hippocampus regulates cocaine CPP, we used replication-defective HSV viral vector to overexpress MEF2 in hippocampus during- and post-conditioning. The CPP protocol was modified to fit the 4-5 day expression time-course using HSVs. Pilot studies suggested that 2 training sessions were sufficient to produce robust CPP in hybrid (C57B1/6 x 129Svev) mice. Two conditioning sessions were conducted for 2 consecutive days (morning: saline; afternoon: cocaine) separated by at least 6 hours. In this set of experiments, we investigated the role of MEF2 in hippocampus at different stages of contextual drug-associated memories. The experimental design is depicted as follows:

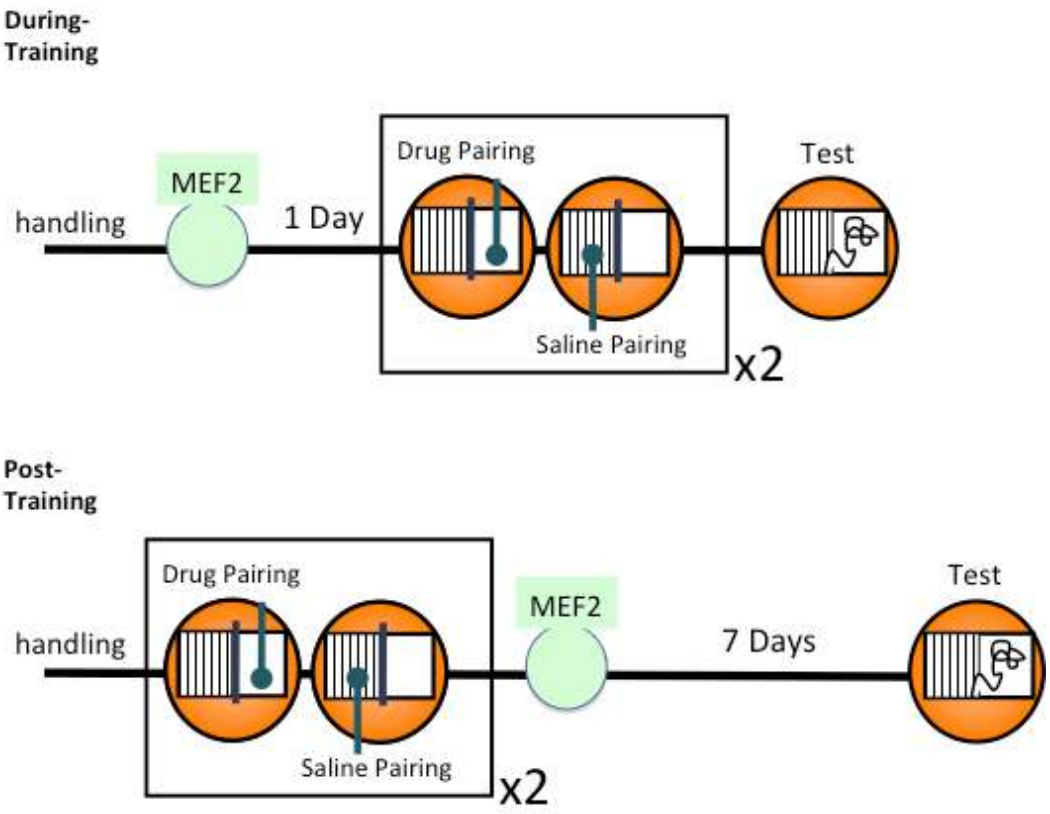


Figure 4.28 Experimental design to examine the role of MEF2 in cocaine associated contextual memory.

4.5.1 Overexpression of MEF2 during training impaired CPP

All the training and test procedures were described in materials and methods. Tests were performed seven days after surgery, according to the protocol modified from previous research (Vetere et al., 2011). As above, movement of animals was monitored, and time spent in the saline-paired and cocaine-paired compartment was computed.

We observed that mice microinjected with HSV vector expressing the control GFP showed robust CPP compared to mice microinjected with HSV vector expressing MEF2 (see Figure 4.29). An ANOVA with *Vector* (MEF2 versus GFP) as a between-subject factor and *Side* (cocaine-paired side versus saline-paired side) as a within-subject factor showed a *Vector* x *Side* interaction [$F(1,19)=8.68$, $P<0.01$], indicating that place preference differed in mice that received MEF2 versus GFP. Main effect of *Side* [*Side:* $F(1,19)=39.75$, $P<0.001$; *Vector:* $F(1,19)=3.266$, $P>0.05$] was also observed, indicating that mice in general spent more time on the cocaine-paired side during testing. Time spent in cocaine-paired versus saline-paired sides was compared using paired *t*-tests in both groups. Paired *t*-test revealed that whereas mice with GFP vector spent more time on the cocaine-paired side versus saline-paired side [$t(8)=21.72$, $P<0.001$], mice overexpressing MEF2 in the hippocampus spent equivalent time on either side [$t(11)=1.98$, $P>0.05$]. Collectively, these data indicate that the overexpression of MEF2 in the hippocampus impaired the formation of cocaine induced CPP. Importantly, there was no significant effects on the distance travelled between MEF2 and GFP groups during the test (unpaired *t*-tests [$t(19)=-0.79$, $P>0.05$], Figure 4.29).

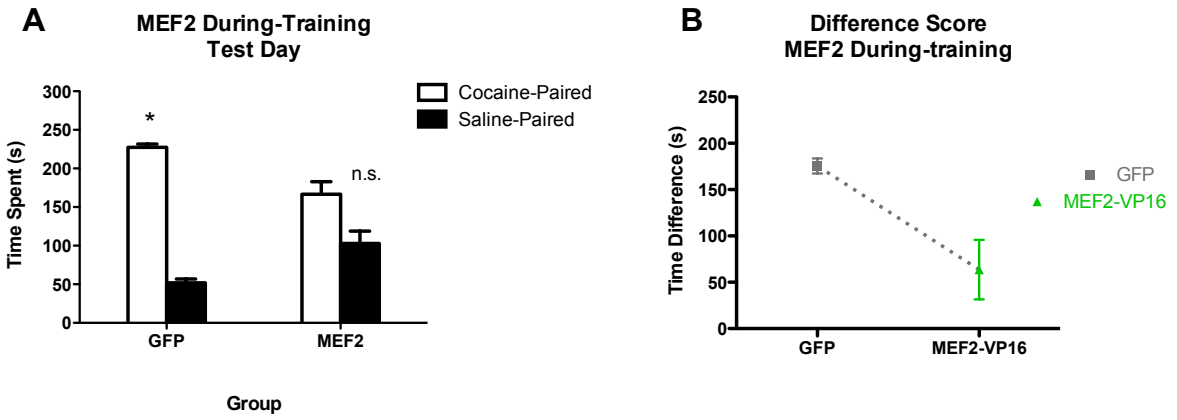


Figure 4.29 Over-expression of MEF2 in the hippocampus during training disrupts formation of a cocaine CPP

A. Time spent for cocaine- and saline-paired sides on a test day in mice that overexpressed MEF2 or GFP in the hippocampus during training. Mice with GFP control vector significantly preferred the cocaine-paired side. In contrast, mice with MEF2 overexpression spent equal time in cocaine- and saline-paired sides (P <0.01, by ANOVA, *Vector* x *Side* interaction), suggesting overexpressed MEF2 during training impaired cocaine CPP memory.

B. Difference scores for MEF2 during-training group showed significant difference between GFP controls versus MEF2 vector. Data are presented as mean ± SEM. *p< 0.05, **p < 0.01, ***p< 0.001 by paired t-test.

4.5.2 Increasing MEF2 post-training did not impair CPP

All the training and test procedures were described in materials and methods. Tests were performed seven days after the surgery, according to the protocol modified from the previous research (Vetere et al., 2011). Movement of animals was monitored, and time spent in the saline-paired and cocaine-paired compartment was computed.

We microinjected mice with MEF2 or GFP vector following CPP training. On a subsequent test, we observed no difference between the vector groups (see Figure 4.30). An ANOVA with *Vector* (MEF2 versus GFP) as a between-subject factor and *Side* (cocaine-paired side versus saline-paired side) as a within-subject factor showed no *Vector* x *Side* interaction [$F(1, 20)=0.24$, $P>0.05$], indicating that the place preference was similar in the MEF2 versus GFP group. Again, there was no significant effect on the distance travelled between MEF2 and GFP groups (unpaired *t*-tests) [$t(20)=-0.55$, $P>0.05$] (Figure 4.30). Collectively, these data suggest that overexpression of MEF2 in the hippocampus after conditioning did not impair cocaine induced CPP.

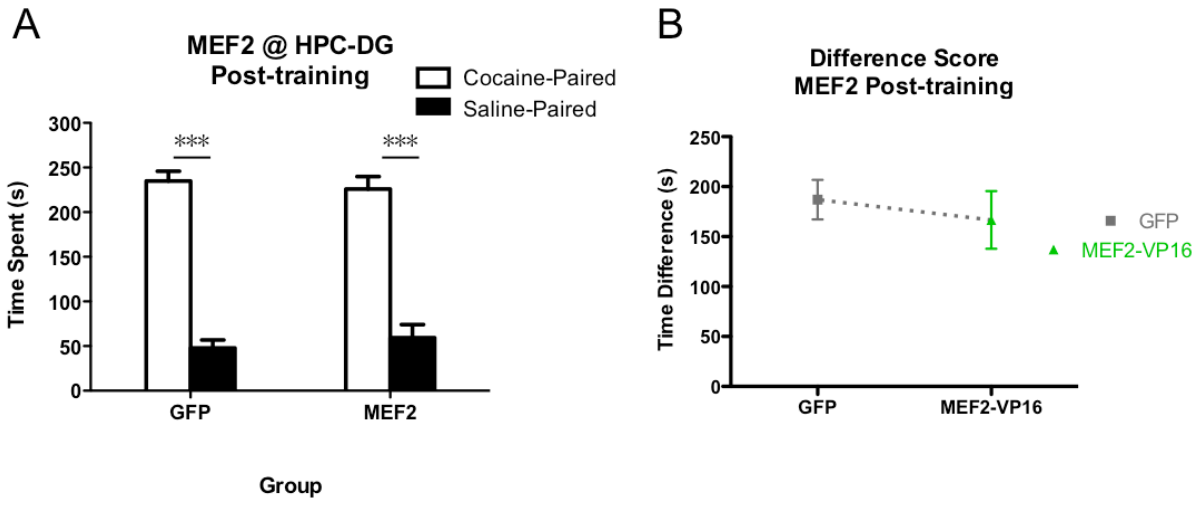


Figure 4.30 Overexpression of MEF2 in the hippocampus after training does not affect subsequent cocaine CPP

A. Time spent in the cocaine- and saline-paired compartment on test day in mice microinjected with MEF2 vector or GFP vector after training. Mice with both GFP control and MEF2 vector showed significant preference for the cocaine-paired side (*P* >0.05 by ANOVA, no *Vector* x *Side* interaction), suggesting overexpressed MEF2 post training did not impair cocaine CPP memory.

B. Difference scores for MEF2 post-training group showed no significant difference between GFP control versus MEF2 vector. Data are presented as mean ± SEM. *p< 0.05, **p < 0.01, ***p< 0.001 by paired t-test.

MEF2 Histology

Placement and extent of viral infection for each mouse was determined using GFP-immunofluorescence by another examiner blind to the behavioral data. Figure 4.31 shows the placement and spread of vector infusions, as determined by highest GFP expression. Only mice that showed robust bilateral expression of GFP in dentate gyrus were included in statistical analysis.

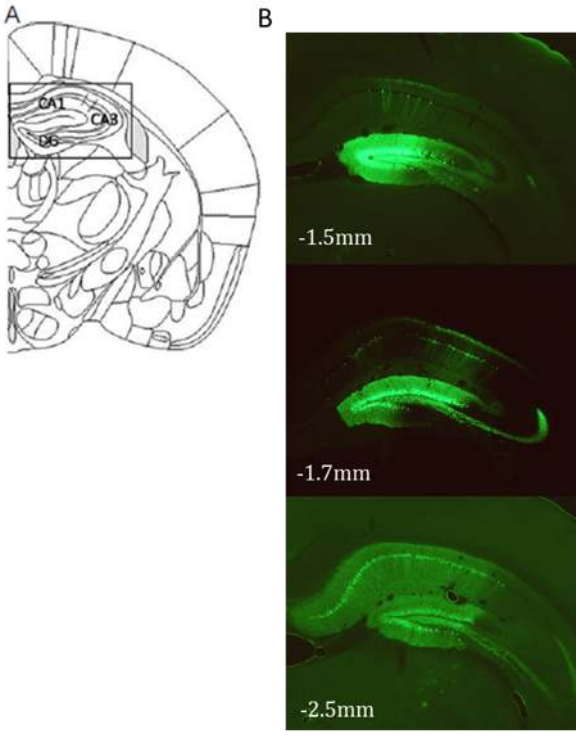


Figure 4.31 Placement of MEF2 vector infusions

A. Target brain region of MEF2 infusion
B. Robust, localized transgene expression of GFP following infusion in the hippocampus.

4.6 Summary

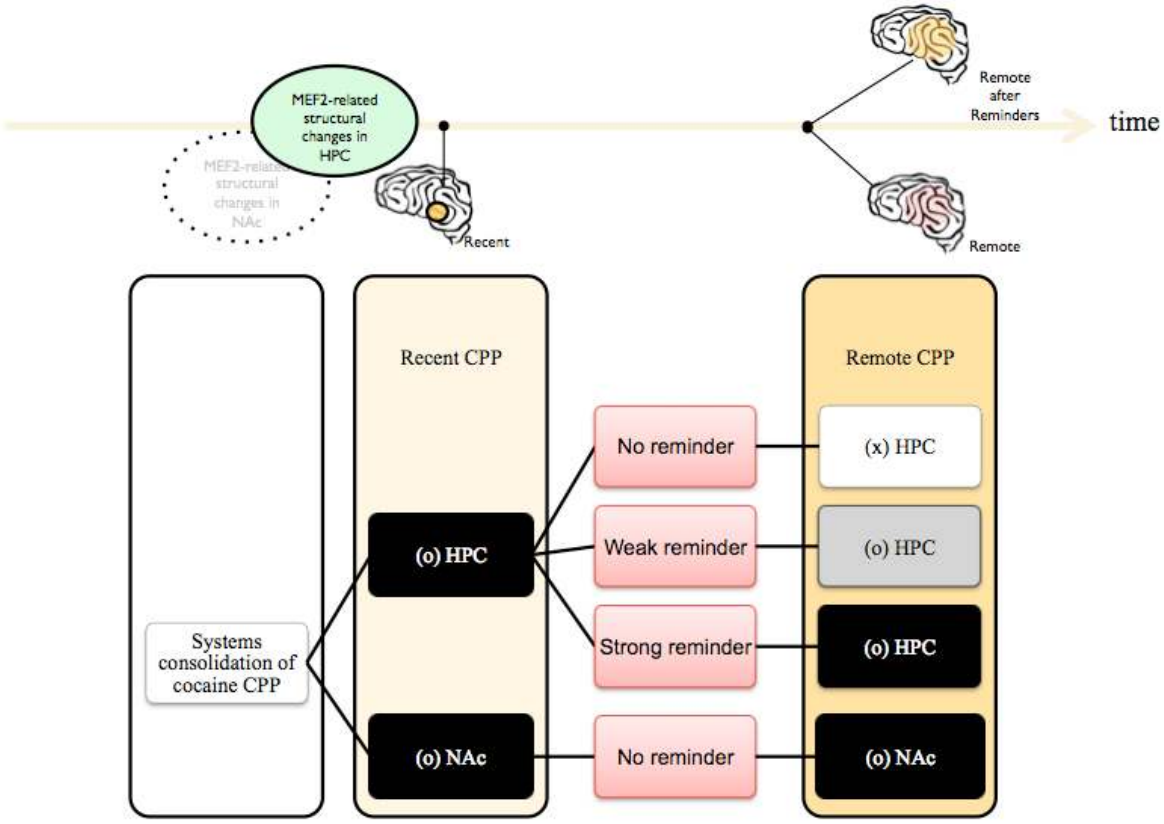


Figure 4.32 Summary of reorganization of CPP memory

Hippocampal lesions disrupt recent (1-day old), but not remote (30-day old) cocaine-CPP memory, while nucleus accumbens lesions disrupt both recent and remote cocaine-CPP memory. Moreover, remote cocaine CPP memories become hippocampal-dependent after reactivation by reminders. The transcription factor, MEF2, is involved in the formation of a cocaine CPP memory but not in its expression.

(o) = Involvement in cocaine CPP.

5 Discussion

5.1 Summary of results

Previous results have shown that in patients and experimental animals that hippocampal damage produces a larger memory impairment for memories acquired recently before the damage and a lesser memory deficit for memories acquired a long time before the damage (a temporal gradient of retrograde amnesia) (Squire & Alvarez, 1995; Frankland & Bontempi, 2005; Kim & Fanselow, 1992; Squire & Chace, 1975; Squire et al., 1975). The current study investigated whether hippocampal damage would also produce this type of temporally-graded retrograde amnesia in contextual cocaine-associated memory in mice. To examine contextual cocaine-associated memory in mice we used the CPP paradigm. Much is known about the time-dependent effects of consolidation for contextual fear memory, however, very little is known about systems consolidation and reconsolidation for contextual drug-associated memory. Given the critical part that memory consolidation might play in the relapse to drug addiction (Hyman et al., 2006), the current study is designed to systematically explore systems consolidation and reconsolidation in cocaine-associated memory, referring to existing frameworks for the time-dependent reorganization of memory in contextual fear studies.

In the first set of experiments, we examined the role of two different brain regions in the consolidation of a cocaine-induced CPP. We first examined the role of hippocampus in cocaine-induced CPP at different time points after conditioning. Mice were trained to acquire cocaine CPP in the place preference apparatus and randomly assigned into sham or lesion groups. Either one day (recent group) or 30 days (remote group) later, mice received NMDA-induced lesions of the hippocampus (or sham surgery), and cocaine place preference was evaluated 10 days later. At the recent time-point (1 day following training), hippocampal lesions impaired cocaine CPP. In contrast, at the remote time-point (30 day following training), hippocampal lesions did not affect cocaine CPP memory. Our data suggest that hippocampal lesions impair recent, but not remote cocaine CPP, memory. This pattern of temporally graded retrograde amnesia following hippocampal damage is strikingly similar to the memory deficits induced following contextual fear conditioning (in which hippocampal lesions produce a more profound amnesia when administered shortly after training) (Kim & Fanselow, 1992). We also examined the effects of lesioning the NAc, an important brain region for reward and cocaine-induced behavior (Pierce

and Kumaresan, 2006), at different times following cocaine conditioning. Unlike hippocampal lesions, we noted NAc lesions impaired both recent and remote cocaine CPP. These findings suggest that the NAc does not play a time-dependent role in cocaine CPP, but instead is always involved (at both recent and remote time points). Therefore, the hippocampus, but not the NAc, plays a time-limited role in systems consolidation of a cocaine CPP memory.

In the second series of experiments, we examined systems reconsolidation in cocaine CPP. It has recently become appreciated that similar to initial memory consolidation after training, established memories might become labile after a reminder and undergo subsequent re-storage or reconsolidation (Nader et al., 2000). This reconsolidation has been shown not only at the level of the molecular mechanisms underlying memory formation, but also at the systems level. That is, an established hippocampus-independent memory may become dependent on the hippocampus once again following a reminder (Debiec et al, 2002). We asked whether the cocaine CPP memory, similar to a contextual fear memory, also undergoes this type of systems reconsolidation following a reminder. In the present experiments, we adopted a similar experimental framework as used in contextual fear memory studies (Winocur et al., 2009). Specifically, we used three different modalities of reminders following initial training: we reminded the mice using either a drug (cocaine)+context reminder (CS+US), drug alone reminder (US), and context only (CS) reminder. To examine whether these reminders re-engaged the hippocampus 30 days following training, we gave mice hippocampal lesions. Importantly, lesions of the hippocampus at this time-point in mice that are not reminded (see above) have no effect. We found that exposure to the drug+context reminder one day before the hippocampus lesion surgery disrupts remote cocaine CPP. This suggests that systems reconsolidation was engaged by the reminder. These findings agree with previous data collected in contextual fear conditioning, suggesting that reactivations of a remote (hippocampal-independent) memory by reminders re-engage the hippocampus (Debiec et al, 2002; Winocur et al., 2009).

The last set of experiments explored how structural changes in the hippocampus modulate the cocaine CPP memory. MEF2 is a transcription factor that negatively regulates spine growth in an activity dependent manner both *in vitro* and *in vivo*. It has been previously shown that locally and acutely increasing MEF2 function in the hippocampus impairs spatial memory formation while decreasing MEF2 function facilitates spatial memory formation (Vetere et al, 2011; Cole et al, 2012). These bi-directional effects suggest that MEF2 function is important in the structural

plasticity underlying memory formation. In the current study, we investigated whether MEF2-mediated structural plasticity in the hippocampus also regulates cocaine CPP memory. To this end, we used replication-defective HSV viral vector to overexpress MEF2 in hippocampus. Our results show that overexpression of MEF2 during training impairs cocaine CPP memory. This observation is consistent with previous studies showing that similarly increasing MEF2 function inhibits memory following contextual fear or watermaze training (Cole et al., 2012). Together, these findings indicate that similar molecular and structural processes may mediate formation of a fear, spatial or cocaine CPP memory.

5.2 Systems consolidation of conditioned place preference

5.2.1 Systems consolidation in contextual fear memories

Systems consolidation refers to a process of memory reorganization - initially certain types of memories (spatial, contextual) are encoded in the hippocampus then these memories slowly reorganize in the cortex and other brain regions. Kim and Fanselow (1992) reported that rats receiving hippocampal lesions 1 day after contextual fear conditioning (the recent group) displayed impaired contextual fear memory, whereas rats that received similar lesions 28 days after conditioning (the remote group) displayed freezing levels equivalent to sham animals. These findings indicate that disrupting hippocampal function preferentially affects recent, but not remote, contextual fear memories (Kim & Fanselow, 1992). These sorts of studies are taken as evidence that these initially hippocampal-dependent memories reorganize over time.

A similar temporally-limited role of the hippocampus has been demonstrated in different versions of contextual fear conditioning and several other behavior paradigms (Anagnostaras et al., 1999; Debiec et al., 2002; Maren et al., 1997; Quinn et al., 2008; Ross & Eichenbaum, 2006; Wiltgen & Silva, 2007; Winocur et al., 2007). For example, Anagnostaras and colleagues (1999) used a within-subject design to show that with the same cohort of animals, hippocampal lesion impaired only recently, but not remotely acquired, contextual fear memories (Anagnostaras et al., 1999). Disruption of hippocampal function produces a similar pattern of results (referred to as a temporally-graded retrograde amnesia) in other types of memory paradigms, including spatial discrimination (Cho et al., 1993; Maviel et al., 2004), visual discrimination (Wiig et al., 1996), trace eyeblink conditioning (Kim et al., 1995; Takatsuki et al., 2002), object discrimination (Zola-Morgan & Squire, 1990), and inhibitory avoidance (Quillfeldt et al., 1996). Together, these data suggest that the neural circuits supporting memory undergo reorganization in a time-dependent manner (Akers & Frankland, 2009; Frankland & Bontempi, 2005; Winocur et al., 2010).

Interestingly, flat gradient retrograde amnesia (i.e., both recent and remote memories are equally affected by hippocampal damage, at least for the period examined) is also reported. For example, some studies found that hippocampal lesions impaired both recent and remote spatial memory in the water maze (Sutherland et al., 2008). Several theories have been proposed to explain the

discrepancy with regard to the nature of the retrograde amnesia (flat or temporally-graded). For instance, it has been proposed that in spatial memory, the hippocampus is always required for its role in integrating spatial details and/or navigation to perform the task (Whishaw, 1998). To be specific, some propose that the hippocampus is required for the path integration and updating of the spatial cues in the environment. Therefore, without an intact hippocampus, mice could not navigate and perform in the spatial memory tasks (and hence one would expect a flat gradient). Another potential factor is the extent of lesions (Akers & Frankland, 2009). In many studies that reported an amnesia with temporal gradient used partial hippocampal lesions (e.g. Kim et al, 1992), while in the studies where the gradient was absent (flat), more extensive hippocampal lesions were employed (e.g. Gaskin et al, 2003). However, spared remote memories were also found after extensive hippocampal lesions in some studies (e.g. Wang et al, 2009) and flat gradients were found after partial hippocampal lesions as well (Martin et al, 2005). Variations in stimulus modality required for learning might also influence whether a gradient is observed. For example, when there is a significant odor involved in the memory component, such as social-transmitted food preference memory, retrograde amnesia with a gradient is consistently found (Ross & Eichenbaum, 2006). It also has been proposed that a prominent odor is often found in the contextual fear memories where the gradient is found (Sutherland et al, 2008). Despite all these findings on the stimuli modality's connection to the presence of gradient, more research is still required to clarify the factors and mechanism regulating the length of gradient and how hippocampus is involved.

In the current study, cocaine-induced CPP paradigm was chosen to study the drug-associated contextual memory because this paradigm shares many common features with contextual fear conditioning. For example, both CPP and contextual fear memory are highly context-dependent memories. Specifically, the context of CPP includes olfactory, visual, tactile, time and many other components. In our study, acetic acid (olfactory), different patterns of walls (visual) and different floors (tactile) stimuli were used to distinguish the cocaine- versus saline-paired contexts. Consistent with the studies suggesting that the hippocampus plays an important role in contextual fear memory, the present study further demonstrated the importance of hippocampus in the process of systems consolidation in cocaine CPP memories.

5.2.2 Temporal gradients in contextual discrimination tasks

Another important factor that may influence whether the retrograde amnesia has a temporal gradient is the context dependency of the behavior paradigm used. Interestingly, two major theories of memory systems consolidation predict different roles of the hippocampus in a remote context-dependent memory. The standard systems consolidation theory states that a remote memory could survive hippocampal disruption, regardless the contextual dependence of memory (Squire, 2004). However, the multiple trace theory predicts that while a context-free memory (semantic memory) could survive hippocampal damage at remote time-points, context-dependent memory (episodic memory) would still be hippocampus-dependent at remote time points (Nadel & Moscovitch, 1997; Rosenbaum et al., 2001).

Indeed, the hippocampus is implicated in maintaining the precision of context in memories; therefore, discrimination tasks (such as CPP) might be a more sensitive task with which to study the role of the hippocampus rather than a simple context fear conditioning task. Studies of contextual fear conditioning have shown that the hippocampus is critical for the contextual component of fear memories (Morris, 2006; Selden et al., 1991). For example, Selden et al. (1991) examined the "place preference" for conditioned fear. In Selden's study, when two contexts were presented (one was black, paired with a shock; the other was brightly lit with white walls, not associated with shock), the safe context was preferred during the test. Importantly, hippocampal lesions before training selectively impaired this contextual association in the preference test (Selden et al., 1991). Moreover, the hippocampus is reported to have a unique role in the discriminative contextual fear memory (Frankland et al., 1998; McDonald et al., 2007; Otto & Poon, 2006; Parsons & Otto, 2008; Wang et al., 2009), rather than in the contextual fear memory tasks that do not have a discrimination component. In fact, it has been suggested that the role of the hippocampus is different in the systems consolidation of discriminative versus non-discriminative contextual conditioning memory (Parsons & Otto, 2010). Indeed, consistent with the multiple trace theory, some studies have shown that fear conditioning and food preference memories lose contextual specificity as they age and become independent from the hippocampus at remote time points (Wiltgen & Silva, 2007; Winocur et al., 2007). However, hippocampal participation in the maintenance of discriminative contextual conditioning appears to be temporally graded in some studies (Parsons & Otto, 2010; Wang et al., 2009). This is somehow inconsistent with the multiple trace theory, because discriminative,

context-specific memories (memories tied to the context in which they were encoded) remain intact when the hippocampus is disrupted at a remote time point. Indeed, it is still not clear how hippocampus involves in the systems consolidation of a discriminative, contextual drug-associated memory.

Data from the present study provide additional evidence for systems consolidation of discriminative context memories. Standard consolidation theory predicts that remote memories would survive hippocampal damage (Squire, 2004). In contrast, the multiple trace theory predicts that only context-independent memories (semantic memories) survive hippocampal damage; context-dependent memories, on the other hand, always require the hippocampus and are qualitatively different from hippocampus-independent memories. Wang et al. (2009) reported that hippocampal lesioned mice are still able to distinguish a remotely-trained context from a similar but different context, suggesting that expressing a remote, precise discriminative contextual fear memory does not require the hippocampus. Although extensive hippocampal lesions made 1 day after training abolished discrimination between the two contexts, lesions made 42 days after training did not impair this context-specific memory. This protocol is similar to the CPP training protocol used in the current study in that the mice were also trained in context A (paired with cocaine) and context B (paired with saline). The findings from the present study are consistent with those from Wang et al. (2009) and also support the idea that a context-dependent and context-specific memory may not always require the hippocampus. Since CPP by nature is a contextual discriminative phenomenon, this may not be surprising. Collectively, these data suggest that extra-hippocampal systems can support context-specific memories to a certain degree, at least under some circumstances. Indeed, it has been proposed that the amount of pre-surgical conditioning may correlate with the dependency on hippocampus at the remote time point (Wang et al., 2009; Akers et al., 2009). However, even if mice could still distinguish between two contexts, the strength and the robustness of the contextual component of the cocaine CPP memories in hippocampal lesion mice remains unclear and further experiments are required to more thoroughly understand this point. More discussion about the role of hippocampus in context representation is presented in the reconsolidation section (below).

5.2.3 Temporal gradients in drug-associated contextual memories

It is not yet clear whether drug-associated memories undergo reorganization over time (systems consolidation). Though it has been suggested that memory may play a critical role in drug addiction and relapse (Muller, 2013; Hyman, 2005), the systems consolidation in cocaine contextual memories has not been systematically studied. There are, however, several lines of evidence supporting the idea that neurobiological substrates supporting drug-associated contextual memories do change over time.

Behaviorally, it has been shown that drug-related cravings elicited by presentation of a context previously associated with drug administration, increases as a function of time. This "incubation" phenomenon has been shown in both cocaine self-administration and cocaine CPP, and suggests that context-elicited cravings for cocaine increases over a period of abstinence (Grimm et al., 2001; Thomas et al., 2008; Tran-Nguyen et al., 1998). Clinically, repetitive reflection on drug-related memories in humans predicts future increases in substance abuse symptoms (Nolen-Hoeksema et al., 2007). This incubation has been reported not only in cocaine, but also in methamphetamine (Shepard et al., 2004), alcohol (Bienkowski et al., 2004), nicotine (Abdolahi et al., 2010), and sucrose self-administration (Grimm et al., 2005).

Neurobiologically, it has been shown that time-dependent neurobiological alterations are induced by drugs of abuse, which might account for the incubation phenomenon, can be found in several brain regions, including DG (dentate gyrus) in hippocampus (Chauvet et al., 2011; Hearing et al., 2010), NAc (Harris et al., 2007; Neisewander et al, 2000) and amygdala (Lu et al., 2005, 2007). Moreover, there is also a time-dependent change in the expression profile of different IEGs produced by either context or cue exposure previously paired with drugs of abuse. For example, context/cue induced reinstatement is not associated with IEG expressions in NAc shell, central amygdala and BNST after a short period of withdrawal (Miller and Marshall, 2004, 2005); however, after several weeks of withdrawal period, significant activation of these regions has been reported (Chauvet et al., 2011; Zavala et al., 2007). Collectively, these data support the idea that systems consolidation found in contextual fear conditioning might also be found in drug-associated memories; the circuits supporting drug-associated contextual memories may change as a function of time.

Contextual conditioning for cocaine is reported to be hippocampus-dependent. For example, it has been shown that the hippocampus exhibits neuronal activation associated with cocaine-seeking behavior in a previously drug-paired environment (Neisewander et al., 2000). Lesions and temporary inactivation of the hippocampus with muscimol before training and before testing impaired cocaine CPP (Hernández-Rabaza et al., 2008; Meyers et al., 2003; Meyers et al., 2006). Fuchs et al. (2005) also reported that application of tetrodotoxin (a sodium channel blocker which would act as a temporary inactivation) in the dorsal hippocampus attenuated context-induced drug reinstatement (Fuchs et al., 2005). Moreover, both the IEGs zif268 and arc are transiently induced in the hippocampus by cocaine-associated stimuli (Hearing et al., 2010; Zavala et al., 2008). However, these studies do not clarify whether the hippocampus is always required at different time points of abstinence for cocaine-associated memories. In addition, the correlational studies may not directly reflect the necessary role of the hippocampus in drug-associated memories and how it changes over time.

The current study is one of the first to systemically examine the role of the hippocampus at different time points after drug-conditioning. Our data provide evidence suggesting a relationship between hippocampal function and the expression/ reorganization of contextual cocaine-associated memory at different time points after training. The preference for the cocaine-paired side in remote, but not recent, CPP following hippocampal lesion indicates that contextual cocaine conditioned memory becomes less hippocampus-dependent over time.

There might be other potential factors cofounding this finding. First, it is possible that the hippocampal lesions in the remote group were sufficiently large to produce a behavioral effect. To address this, we assessed performance in a known hippocampal-dependent task the water maze (Morris et al, 1982), in the same mice. We found that in the same mice with hippocampal lesions (which did not produce a remote memory deficit for cocaine-CPP), showed impairment in water maze learning. These findings verify that the hippocampal lesions were sufficient to produce a behavioral effect. Second, the CPP impairment in the recent group may simply have been due to a non-specific locomotion effect. Indeed, one of the behaviors produced by hippocampal lesion is hyperactivity, which may interfere with the expression of place preference. However, it is unlikely that the CPP impairment in the present study is due to hyperactivity, since lesioned mice in the remote group exhibited similar amount of increased locomotor activities but still showed a preference for the cocaine-paired side. As the only variance between

the recent versus remote group is the waiting period between training to surgery, all other procedures between lesion surgeries to testing remained the same. Therefore, the CPP impairment in the recent group is unlikely due to nonspecific surgery-induced effects.

The data from the current study is in line with the previous studies and further demonstrate the role of hippocampus in cocaine CPP expression. In the study by Meyers et al. (2006), the researchers showed that temporal inactivation of the hippocampus with the GABA-A agonist muscimol blocked the acquisition and expression of cocaine CPP in rats 1 day after training. The idea that the hippocampus is essential for drug-context association is further supported by the data collected in cocaine reinstatement studies using self-administration. For example, Fuchs et al. (2005) showed that hippocampal inactivation specifically disrupts contextual cocaine-seeking behavior, but not drug-primed reinstatement of cocaine-seeking behavior, suggesting that the hippocampus is essential in creating the drug-context association (Fuchs et al., 2005). Along with the current data, since hippocampal lesions at remote time points did not impair cocaine CPP, it is possible that the association between context and drug experience may be supported by the brain regions outside the hippocampus at a remote time points, similar to the findings in contextual fear conditioning (Wang et al, 2009).

An extensive literature has demonstrated that the hippocampus is involved in contextual conditioned and unconditioned stimuli (CS–US) associations in aversive US (Kim et al., 1993; Maren et al., 1997;). Here we show that a time-dependent role of the hippocampus in the context-dependency of an appetitive memory. In conclusion, the current study contributes to a growing literature exploring the role of the hippocampus in the reorganization of cocaine-associated memory circuits.

5.2.4 Nucleus accumbens and cocaine CPP

Anatomically, the nucleus accumbens (NAc) is a major terminal region of the mesolimbic dopamine system projecting from the ventral tegmental area (VTA) and medial substantia nigra pars compacta (SNc), amygdala, bed nucleus of stria terminalis, lateral septal area, lateral hypothalamus, and olfactory tubercle. Additionally, the NAc sends projections not only to basal ganglia structures, such as the globus pallidus, entopeduncular nucleus, and motor thalamus, but also to limbic structure, such as the extended amygdala and the lateral hypothalamus.

As the mesolimbic dopamine pathway is implicated as the reward pathway, the NAc is therefore considered an important component of drug-associated behavior, such as conditioned place preference, self-administration, and increased locomotor activity (Pierce and Kumaresan, 2006; Wise, 2004). In fact, the involvement of NAc in drug-associated behaviors has been demonstrated in many classes of drug abuse, such as psychostimulants (Lyness et al., 1979; Di Chiara & Imperato, 1988), opiates (Di Chiara & North, 1992), ethanol (McBride et al., 2002), cannabinoids (Ameri, 1999) and nicotine (Singer et al., 1982). This dissertation will briefly discuss the importance of the NAc in mediating physiological effects of cocaine and cocaine-induced behavior such as locomotor sensitization, self-administration, and conditioned place preference (Wise, 2004; Pierce and Kumaresan, 2006).

Locomotor sensitization refers to the phenomenon that repeated exposure to cocaine results in a long-lasting enhancement of the behavioral response (locomotion). Locomotor sensitization can be observed after several weeks, months, or up to at least a year of drug-free period (Robinson and Berridge, 1993; Vanderschuren & Kalivas, 2000). Lesion and pharmacological manipulation have implicated the NAc in cocaine sensitization (Robinson and Berridge, 1993; Baik, 2013). Repeated administration of cocaine into the NAc has been reported to cause sensitization (Hooks et al. 1993). Moreover, sensitized animals show enhanced DA release in the NAc following drug exposure (Kalivas and Duffy, 1990). For the long-term expression of behavioral sensitization, the NAc continues to play an important role of this behavior, as structural and functional neuronal changes are found in the NAc (Wolf et al., 1993; Paulson and Robinson, 1995; Robinson and Kolb, 2004).

Cocaine is self-administered in many animals (Pickens and Thompson, 1968). Various changes of the NAc affect self-administration differently. Bilateral 6-hydroxydopamine lesions in the NAc produced a significant impairment of cocaine self-administration (Roberts et al, 1977, 1980; Pettit et al, 1984; Gerrits and van Ree, 1996). Dopamine receptor antagonist infusion in NAc also reduced self-administration of cocaine (Maldonado et al, 1993; Suto et al, 2009). Collectively, these studies implicate that the NAc in cocaine reward or reinforcement.

As for the conditioned place preference, it has been shown that pre-training bilateral 6-hydroxydopamine lesions of the NAc shell impair the acquisition of intravenous cocaine CPP (Sellings, 2006). Consistent with this, lesions of the NAc also impair the acquisition and

expression of another psychostimulant (amphetamine)-induced CPP (Sellings, 2003). Moreover, evidence from pharmacological intervention studies shows that pre-training and pre-test inactivation of the NAc with the glutamate antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) impairs the acquisition and expression of cocaine CPP (Kaddis et al., 1995). Collectively, these data suggest that both glutamatergic and dopaminergic pathways in the NAc are critical in cocaine CPP. This is consistent with the data from self-administration studies, which suggest that systemic administration of dizocilpine (or MK-801, an NMDA receptor antagonist) reduces cocaine self-administration (Pierce et al., 1997).

While it is known that the NAc is involved in cocaine CPP, cocaine self-administration, and long-term expression of cocaine-induced sensitization, it is not entirely clear that how the NAc is implicated in the expression of a recent versus a remote cocaine contextual memory. However, there is some evidence indicating that the NAc is involved in cocaine-induced contextual behavior even long time after abstinence. For example, a few studies have showed that the NAc is one of the critical brain regions in the incubation of drug craving (Pickens et al., 2011). Moreover, time-dependent neurobiological changes are found in the NAc after cocaine exposure (Harris et al., 2007; Neisewander et al, 2000). IEG studies also reveal that neural activities are increased in the NAc after expression of a remotely-formed cocaine-induced contextual CPP and self-administration (Miller and Marshall, 2004, 2005; Chauvet et al., 2011; Zavala et al., 2007).

The current study contributes to the growing literature of the role of the NAc in cocaine CPP at different time points after abstinence. Specifically, our data have shown that the NAc lesions induced by NMDA 1 day and 30 days after training disrupted an established cocaine CPP, suggesting that the NAc is critical for both recent and remote cocaine CPP memory expression. This observation is in line with our hypothesis that the gradient of retrograde amnesia is specific to hippocampal damage, but is not observed in the NAc lesioned animals. The finding that the NAc is required for the expression of a remote (30-day old) cocaine CPP is also consistent with the study by Hollander et al. (2007), which reports that cocaine-associated contextual stimuli cause an increased activation in the accumbens core after 1-month abstinence from cocaine self-administration.

CPP is a task based on the principles of classical conditioning, wherein a neutral environment is paired with the rewarding effects of cocaine (Bardo and Bevins, 2000). Therefore, the NAc could

be important in one of several aspects of this procure. Indeed, in CPP, animals form the association between the context (CS) and the rewarding properties of the drug (US) at training stage. At test stage, animals first recognize the context (CS), and then exhibit the conditioned approach behavior (CR) (Bardo and Bevins, 2000). Therefore, failure to express an established CPP could possibly due to a mnemonic effect (i.e. memory for the context, CS), a reward effect (effects on US; US can not elicit response) or a context-reward association. Sellings et al. (2003) reported that 6-OHDA-induced lesions in the NAc impair amphetamine, but not morphine, CPP, suggesting that the general mnemonic functions in the NAc lesion animals are preserved. Indeed, previous studies have shown that dopaminergic and glutamatergic pathways in NAc play a critical role in cocaine reward-related behaviors, such as locomotor sensitization, cocaine seeking and cocaine self-administration (Baik, 2013; Saunders et al., 2013 and Pickens et al., 2011). Therefore, it may be that the NAc is essential for the rewarding effects of cocaine CPP, and it may follow that lesions of the NAc produce deficits because the conditioned rewarding prosperities of the CS are not processed. From our data alone, we conclude that the NAc plays a critical role in both recent and remote cocaine CPP expression, and further studies are required to clearly define the role of NAc in the reward and conditioning behind CPP.

It is also worth noticing that in this study, lesions in the NAc were not intended to differentiate between the core and shell within the NAc. Evidence suggests that the core and shell subregions of the NAc have distinct functions (Di Chiara et al., 2004) and may play different roles in cocaine-induced behaviors (Sellings, 2003; 2006). However, in this study, NMDA-induced lesions were designed to reliably lesion the entire NAc almost completely, thus to provide a clear-cut comparison with the hippocampal lesion data.

To summarize, we showed that hippocampus plays a temporal role in cocaine CPP memory and that it is not required in expressing remote cocaine CPP. In contrast, the NAc is always necessary in expressing cocaine CPP, even 30 days after cocaine conditioning. These data support that systems consolidation can also be observed in the course of drug-induced contextual memory development, as observed in contextual fear conditioning memories.

5.3 Reengagement of the hippocampus

5.3.1 Systems reconsolidations of memories

Similar to memory consolidation, memory reconsolidation may also occur at the cellular and systems level. Protein synthesis has been shown to be important in memory consolidation. That is, immediately after training, local infusion of the protein synthesis inhibitor, anisomycin, disrupts subsequent memory expression. Similarly, infusion of anisomycin locally into the amygdala immediately after the retrieval of a previously trained fear memory impaired subsequent memory expression. However, when anisomycin was infused 6 hours after memory retrial, there was no memory impairment (Nader et al., 2000). Furthermore, memory was normal if anisomycin was infused in the absence of memory retrieval. With a similar approach using contextual fear conditioning, Debiec et al. (2002) showed that protein synthesis in the hippocampus is required for both consolidation and reconsolidation. Again, the retrieval of the original memory was critical for the impairment. From these experiments, it was concluded that a previously consolidated fear memory, when reactivated, returns to a labile state that requires de novo protein synthesis for reconsolidation. This is referred to as cellular reconsolidation. A similar reconsolidation process has also been studied at the systems level.

In systems reconsolidation, Debiec et al. (2002) found that remote contextual fear memory, which is independent of the hippocampus, briefly returns to a hippocampal-dependent state after rats are reminded of the training context. Specifically, lesioning the hippocampus after memory reactivation impaired subsequent performance for the contextual fear memory, even when the reactivation was delayed for 45 days after training (a time point at which the memory is already hippocampus-independent). These data suggests that hippocampus was briefly critical again after the retrieval of memory (Debiec et al., 2002). This reengagement of the hippocampus during memory retrieval is thought to further refine or incorporates new information into the existing extra-hippocampal memory traces (Dudai, 2004; Dudai, 2011; Frankland & Bontempi, 2005). Even though systems reconsolidation has been well demonstrated in contextual fear memories, it is still unclear whether this process takes place in drug-associated memories.

5.3.2 The reconsolidation of drug-associated memories

A cellular reconsolidation process has been suggested to be of use in treating relapse (Milton and Everitt, 2010; Taylor et al., 2009), as the original drug-related memories might be disrupted or interfered with at this stage when they become labile. At the cellular level, different types of memories, including spatial, contextual fear, and appetitive memories become labile again after reactivation (Morris, 2006; Nader, 2000; Sara, 2000). Importantly, such reconsolidation has also been observed in drug-associated contextual memories, as shown by morphine CPP, cocaine CPP, and self-administration of cocaine (Lee et al., 2005; Milekic et al., 2006; Miller & Marshall, 2005; Wells et al., 2011). However, most of the studies focus on the cellular level reconsolidation; to date, the systems reconsolidation of cocaine-induced CPP memory has not yet been well investigated.

Another important aspect related to systems reconsolidation is whether the quality of the memory changes as it transforms from a stable to a labile state after a reminder. As previously described, in the standard view of systems consolidation, the hippocampus is no longer required to retrieve the remote memory (Dudai, 2004; Squire & Alvarez, 1995). Alternatively, the transformation theory states that the expression of the original memory in its precise form may always require the hippocampus (Moscovitch et al., 2005; Winocur et al., 2009; Winocur et al., 2007). In our first set of experiments, we showed that a remote cocaine-CPP memory is not dependent on hippocampus, and in the second set of experiments, we investigated whether the systems reconsolidation can be observed- that is, if the reactivation of this memory could re-engage the hippocampus. We also examined how different reminders affect the memories' dependency on the hippocampus.

The current study provides direct evidence for the systems reconsolidation of cocaine CPP memories and extends the scope of findings in systems reconsolidation with contextual fear conditioning. Our data suggest that after reactivation, a remote hippocampal-independent cocaine CPP memory becomes hippocampus-dependent again. That is, hippocampal lesions completely blocked the CPP in the strong reminder group, in which the mice re-experience both the context and the drug. In the weak reminder groups (context only or drug only), hippocampal lesions produced a mild effect; a preference for the drug-associated context was still observed. In the no-reminder group, the 30-day old cocaine CPP memory was immune to the hippocampal lesion.

These data are in line with the findings of Debiec et al. (2002) and support the systems reconsolidation hypothesis (Nader & Hardt 2009).

Moreover, the present study also addresses several questions in systems consolidation. In line with studies in contextual fear conditioning, our data showed that although the precise context memory may not require the hippocampus at a remote time point, reactivation of the original memory trace might bring the hippocampus-dependent memory back to a dominant status. Notably, the degree of memory abolishment by hippocampal lesions in the drug+context reminder group was the greatest. For the context only or the drug only reminder group, the hippocampal lesions were only able to attenuate the CPP but not to disrupt it. These data further support the finding by Winocur et al (2009), where the remote contextual fear conditioning memory was susceptible to hippocampal lesions only when the memory trace was reactivated by presentation of the original context, but not the "counterfeit" similar context. As the data were interpreted to support the transformation theory, it is argued that similar context reminders were only able to activate the context-general memory trace whereas the original context reminder was able to make the context-specific memory trace dominant again. Our data are consistent with this argument and are the first available data demonstrating it with cocaine CPP paradigm.

Finally, our data also provides evidence revealing that hippocampal lesion after reactivation of remote cocaine CPP memory disrupted cocaine CPP only in the CS+US reminder (i.e. context and drug) group, but the disruption of CPP was not observed in the CS or US alone group (noted that the preference in lesion animals is significantly lower than the control animals). Interestingly, Milekic et al. (2006) demonstrated a similar trend that an established (1 week old) morphine CPP memory in rats could be disrupted by blocking protein synthesis after reactivation by only the context-and-drug (CS-US) reminder, but not the context alone (CS) reminder (Milekic et al., 2006).

The results from the current study are also in line with related studies using the self-administration model. Ramirez et al. (2009) reported that tetrodotoxin-induced neural inactivation of the hippocampus following re-exposure to the cocaine-paired context inhibits the subsequent context-induced reinstatement of cocaine seeking. However, anisomycin treatment in the hippocampus did not produce this effect (Biedenkapp & Rudy, 2007; Ramirez et al., 2009). This suggests that the hippocampus may be not the locus of memory per se, as anisomycin-

sensitive processes are considered to be necessary for memory reconsolidation (Tronson & Taylor, 2007). While the hippocampus may not be the critical site for the protein synthesis and post-translational modification required for memory reconsolidation, the interaction between the hippocampus and other brain regions (where reconsolidation occurs) might be necessary for the expression of memories (Wells et al., 2011). For instance, extra-hippocampal structures and intrahemispheric communication between the basal amygdala and the hippocampus might also play a critical role in regulating the reconsolidation of cocaine-related associative memories (Wells et al., 2011). It is one of the possibilities that why the preference for cocaine-paired context could still be observed after the representation of our context-only or drug-only reminder, but not the context+drug reminder, as the representation of partial context did not make the hippocampal-dependent memory trace entirely dominant and some of remained memories trace were located in other brain regions.

In conclusion, the current study suggests that when the memory was activated with different reminders, part of the hippocampal-dependent circuit is reactivated. Manipulating the hippocampus at this time point inhibits the memory and inhibits further cocaine-seeking behavior. Our results also suggest that some form of context specificity may be retained over long intervals without the hippocampus, and the context-specific and context-general memories represented in different brain structures can coexist, although one or the other may dominate under different conditions.

5.4 MEF2 and the structural changes in hippocampus in drug-associated memories

Memory and drug addiction both induce long-term neuronal structural changes in different brain regions, and these structural changes are thought to underlie the formation, storage, and expression of the behavior. The structure of axons, dendrites, and the synapses connecting them are the physical basis of all neuronal communication. Axons send the output of neurons to diverse targets in various regions, and dendrites integrate several inputs from a variety of sources. These connected circuits are thought to be mediated by structural plasticity and are the neuronal basis of our behavior, including memory and addiction.

It has been proposed that structural changes in synaptic morphology are necessary for memory (Lee & Silva, 2009). Learning is thought to increase new synapses and strengthen existing synapses between relevant neurons (Neves et al., 2008; Hofer et al., 2009; Yang et al., 2009) and protein synthesis is important in this process (Bailey & Kandel, 1993).

Structural changes mediating neuronal communication are also important in addiction, especially in relapse. As shown in the memory studies, an increase in dendritic spine density has been observed in several different animal models of addiction (Robinson & Kolb, 2004). As the dendritic spines in the NAc receive excitatory glutamatergic inputs from the prefrontal cortex and dopaminergic inputs from the VTA (Hyman et al., 2006), it has been proposed that the change in spine density in the NAc neurons may mediate information processing and further mediate addiction behaviors. Specifically, cocaine has been shown to increase spine density in the NAc (Lee, 2006), and the transcription factor MEF2 plays a critical role in this increase (Pulipparacharuvil et al., 2008).

It has been shown that MEF2 restricts dendritic spine growth in an activity-dependent way (Flavell et al., 2006), indicating that MEF2 may affect memory formation. Flavell et al. (2006) showed that increasing MEF2-mediated transcription decreases the number of dendritic spines and excitatory synapses in hippocampal neurons in vitro. Upon neuronal activation, calcium entered the neurons, inducing the activation of the calcium/calmodulin-regulated phosphatase calcineurin, which further dephosphorylated and activated MEF2. When MEF2 was activated, it

promoted the transcription of arc and synaptic ras GTPase activating protein (synGAP), which restricts synapse number.

Pulipparacharuvil et al. (2008) reported that MEF2 blocks the increase in spine density following repeated cocaine administration in the NAc neurons in vivo (Pulipparacharuvil et al., 2008). This indicated that cocaine CPP is modulated by the local MEF2-VP16 in the NAc. Closely related to the current study, Cole et al. (2012) have demonstrated that memory formation is associated with increased phosphorylation of MEF2 and decreased MEF2 protein levels in the hippocampus (Cole et al., 2012). Together, the above studies suggest that MEF2-mediated process may negatively regulate the dendritic spine growth, which further regulates memory formation.

To address whether learning-dependent structural modification in the hippocampus is involved in the cocaine CPP memory, we overexpressed MEF2 in the hippocampus via the herpes simplex virus (HSV). Overexpression of MEF2 *during* training impaired cocaine CPP conditioning. This pattern is consistent with the data collected in contextual fear (Cole et al., 2012), where increasing MEF2 in the hippocampus disrupted contextual, but not tone, fear memory. As expected, overexpression of MEF2 in the hippocampus *after* conditioning did not impair cocaine place preference, consistent with previous studies reporting that post-training microinjection of MEF2 viral vectors did not impair water maze or context fear memory. This suggests that MEF2 specifically disrupts the formation of new memories but has a limited role in modifying pre-acquired memory.

Our data provide evidence indicating that MEF2 in hippocampus plays a role in cocaine CPP formation, further suggesting a general role of MEF2 in memory: the disruptive effect of increased MEF2 on memory formation is neither exclusive to a particular brain region nor to a specific type of memory. This effect has been demonstrated in the hippocampus, amygdala, and NAc in several memory tasks, including cocaine CPP, water maze, and fear conditioning (Cole et al., 2012; Pulipparacharuvil et al., 2008). Moreover, our data demonstrates the importance of the hippocampus in contextual drug-associated memory formation.

Collectively, this set of experiments assessed the role of MEF2 in the hippocampus in drug-associated behaviors. Future experiments are still required to reveal the mechanism behind MEF2 regulation of drug-associated memory.

5.5 Implications

The idea that the inhibition of memory reconsolidation may selectively impair the conditioned behavior memory trace has been proposed and demonstrated in several memory behavioral paradigms (Nader, 2003). Indeed, disrupting the reconsolidation of long-term associative memories has been suggested as a possible treatment for post-traumatic stress disorder, phobias, obsessive-compulsive disorder, and addictive behavior (Diergaarde et al., 2008; Milton & Everitt, 2010; Taylor et al., 2009). However, the effects of current clinical cue-exposure therapy is limited, as relapse remains one of the biggest obstacles for treating drug addiction.

To study relapse, several laboratory animal models have been developed. Renewal, reinstatement, and spontaneous recovery may provoke the drug seeking behaviors again after extinction. That is, the resumption of the conditioned drug-association responses has been demonstrated after reexposure to environmental context, drugs, or simply after a passage of time. Indeed, as current cue-exposure approaches are based on the extinction, generalization to the real world outside the clinic could be difficult (Otto et al, 2007). Because extinction is a new learning process, rather than modifying the original memory trace, the therapy based on extinction could be highly context dependent (Taylor et al, 2009). In contrast, reconsolidation is a process where the original memory trace becomes labile to interference; therefore, it is less context-limited when applied in the clinical therapies.

The present research focuses on the memory component in relapse. With the cocaine-induced CPP paradigm, this study examines the function of the hippocampus in the expression of recent and remote cocaine CPP memory. This study provides the first evidence of systems consolidation and reconsolidation of cocaine-induced CPP, suggesting that hippocampus plays a temporal role in this memory. As systems reconsolidation also takes place in cocaine CPP, it provides additional evidence to understand the role of memory in relapse and incubation of drug craving. Still, future research is required to clearly understand the reorganization of cocaine CPP memory.

6 Conclusions

Memory is a process of receiving, organizing, storing and retrieving information that we receive everyday. How would a memory trace develop over time? Our daily experience suggests that we gradually forget the details of a recent event; however, some old memories seem to remain vivid even months and years later. Studies have reported that old memories are less susceptible to brain damage compared to a recently acquired memory, as referred to as Ribot's gradient (Ribot, 1882). Consistent with this observation, later studies identified that hippocampal damage impairs recent, but not remote, memories, suggesting a temporal gradient of retrograde amnesia. For example, one of the most well known cases of this temporally-graded amnesia was patient H.M. (Scoville & Milner, 1957). Following his bilateral medial temporal lobe removal, H.M. was described to show complete amnesia for the memories within months before the surgery. However, he was able to retain memories from his early childhood. This gradient of retrograde amnesia has been reported not only in numerous hippocampal damage patients, but also in animal models (Squire et al., 1975; Alvarez et al., 1995; Frankland & Bontempi, 2005). For instance, Kim and Fanselow showed that hippocampal lesion impaired recent (<14 days) but not remote (28 days) contextual fear memory in rats, indicating that hippocampus is required for a recent, but not remote contextual fear memory (Kim & Fanselow, 1992). This finding is further supported by immediate early gene studies (Dudai, 2004; Frankland & Bontempi, 2005; Frankland et al., 2004), suggesting that contextual fear memory is initially hippocampus dependent, but gradually becomes more hippocampus independent over time. Although the role of hippocampus in contextual fear memory has been extensively examined, the role of hippocampus at different stages of drug-associated memories remained unclear. Studies have suggested that memory is a key component in relapse (Hyman, 2005; Hyman et al., 2006). Incubation of drug craving elicited by drug-associated context further suggests that circuits supporting drug-associated memory may reorganize over time (Lu et al., 2004; Pickens et al., 2011). However, to this date, it is still unclear whether the drug-associated memories undergo systems reorganization.

The present research systematically examines the systems consolidation and reconsolidation in contextual drug-associated memory with cocaine CPP paradigm, a long-lasting contextual drug-conditioning memory paradigm. In cocaine CPP, mice are trained to pair cocaine with one context, and saline with another context. During the test, mice are given access to both contexts in a drug-free status. The time mice spend in both contexts is recorded and used as an indicator of contextual preference.

In our first series of experiments, we examined whether the hippocampal dependency in cocaine CPP changes over time. With the experimental framework from contextual fear memory studies, we lesioned the hippocampus either 1 day (*recent* group) or 30 days (*remote* group) after training mice with cocaine CPP paradigm. We found that hippocampal lesions impair *recent* cocaine CPP memory. However, in the *remote* group, hippocampal lesion mice showed preference at similar level to the sham mice. These data supports our hypothesis that systems consolidation takes place in cocaine CPP memory, and hippocampus plays a temporal role in it.

In the second group of our study, we examined the systems *reconsolidation* in cocaine CPP memory. Specifically, we tested whether reactivation of remote cocaine CPP by different reminders brought these memories back to being hippocampus-dependent again. In the mice exposed to reminders one day before lesion, hippocampal lesion was able to impair a 30-day-old cocaine CPP memory. These data support our hypothesis that systems reconsolidation can be observed in the contextual drug-associated memories as well, similar to the studies in contextual fear memory.

Third, we further examined whether structural changes in the hippocampus regulates cocaine CPP memory. By overexpressing myocyte enhance factor 2 (MEF2) in the hippocampus either *during* cocaine CPP training or *after*, we showed that MEF2-mediated changes in hippocampus impaired acquisition, but not the consolidation of cocaine CPP memory. This data suggest that MEF2-mediated hippocampus structural changes are critical in the formation of cocaine CPP memory.

In conclusion, the current study demonstrated that systems consolidation and systems reconsolidation also occurs in cocaine CPP, suggesting that circuits supporting contextual cocaine memory reorganize over time.

7 Future Direction

The current study examined the role of hippocampus in contextual cocaine-associated memory (CPP) and how it develops over time. In particular, excitotoxic lesions allowed us to study the causal link between hippocampus and cocaine CPP at different time points after training (i.e., during abstinence). In the first set of experiments, the hippocampus was lesioned either 1 day (*recent* group) or 30 days (*remote* group) after training with CPP paradigm. Our data suggest that hippocampal lesion impaired *recent*, but not *remote*, cocaine CPP memory. This temporal gradient indicates that remote cocaine CPP became less hippocampus dependent over time. In the second set of experiments, we examined the role of hippocampus in systems reconsolidation of cocaine CPP. Thirty days after training, four groups of reminders (CS+US, CS, US, No reminder) were given 1 day before the hippocampal lesion surgery. Our data suggest that, after being revived by reminders, remote cocaine CPP memories become sensitive to hippocampal lesions again. As structural changes are essential for memory formation and consolidation, the last set of experiments was designed to explore how structural changes in hippocampus modulate cocaine CPP memory. Myocyte Enhancer Factor 2 (MEF2) was overexpressed in the hippocampus via herpes simplex virus (HSV) *during*- and *post*-training. We found that overexpression of MEF2 *during* training, but not *post*-training, impaired cocaine CPP. Collectively, our data suggest that hippocampus and its structural changes are critically involved in the cocaine CPP memory.

7.1 Identify the neuronal circuits mediating remote drug-associated memory

One of the most important research questions in memory reorganization/systems consolidation is where remote memories are stored in the brain. Theoretical accounts propose that remote memories are stored in complex, distributed networks. These networks are thought to contain ‘hub’ regions that may play disproportionately important roles in memory expression. Therefore, a future goal would be to identify remote memory ‘networks’ for CPP and ask whether targeting ‘hub’ regions in these networks impacts expression of cocaine CPP at remote time points.

In previous studies in the lab we used a c-fos imaging based approach to identify networks underlying recent and remote contextual fear memory (Wheeler et al 2013). Briefly, mice were

fear conditioned and then given a retrieval test after a 1-day (recent test) or 30-day (remote test) delay. Fos was then quantified in 84 brain regions to identify populations of cells activated during the retrieval test. Then, by computing a complete set of cross correlations we were able to identify collections of brain regions where Fos co-varied, and presumably represent a network of regions that are co-active during memory recall. Graph theoretical analyses of these networks indicated that they were small-world in nature, and contained several highly-connected hub regions (including the hippocampus and ACC).

Performing an equivalent analysis following recall of recent and remote CPP would allow us to identify CPP networks. In the current experiments we found that the nucleus accumbens (NAc) is required for both recent (1-day, hippocampus dependent) and remote (30-day, hippocampus independent) contextual drug-associated conditioned memory. Therefore, these functional data already point to the NAc and hippocampus (at least at recent time points) playing key 'hub-like' roles in these CPP networks. Such a network analysis would also identify several other candidate hub regions, and it would be interesting to evaluate whether targeting these structures (using, for example, excitotoxic lesions or optogenetic interventions) would impact CPP expression in predictable ways.

A second issue is to what degree networks for CPP and fear memories overlap. It is likely that there will be overlap in several core regions. For example, our data suggest that the hippocampus plays a similar (temporally-graded) role in the expression of cocaine CPP and contextual fear memories. Other areas of potential overlap include the ACC (which is implicated in remote contextual fear memory), as well as the amygdala (implicated in expression of both cocaine CPP and contextual fear memories). Where the networks differ will also be informative. These differences should underscore differences in memory content to some degree. For example, it is likelier that regions implicated in processing of the reinforcing properties of drugs such as the NAc would be implicated in CPP memory networks.

7.2 Hippocampus-dependent vs. hippocampus-independent memory traces

One idea in the contextual fear conditioning literature is that while expression contextual fear memories may be mediated by either hippocampus or hippocampus-independent traces, the nature of these memoires differs. In particular, hippocampus-dependent contextual fear

memories are argued to be precise (or contextually-rich) with mice discriminating between the shock-paired context and other similar contexts. In contrast, hippocampus-independent memories tend to be more generic or semantic in nature with mice unable to discriminate between the shock-paired context and other similar contexts. Similar to some previous contextual fear conditioning studies (Winocur et al., 2007; Debiec et al., 2002), the current study suggests that relationship between hippocampus or hippocampus-independent traces for cocaine CPP is dynamic: Simple reminders can return remote (hippocampus-independent) memories back to a hippocampus-dependent state.

One core question here is whether these hippocampus-independent, remote cocaine CPP memory indeed distinct from the hippocampus-dependent, recent cocaine CPP memory. One previous contextual fear conditioning study is especially relevant in this regard. Wang et al (2009) trained mice in a discriminative fear conditioning procedure where one context was paired with shock, and a different context was explicitly not paired with shock. Procedurally this is almost identical to the cocaine CPP study reported here, except that in our case the US was an injection of cocaine rather than a shock. Wang et al (2009) found that hippocampal lesions did not abolish discrimination between the shock-paired and shock-unpaired contexts at the remote time point. We observed a very similar pattern of results with cocaine CPP, with mice able to discriminate the drug-paired vs. drug-unpaired compartments following hippocampal lesions at remote time-points. The sparing of context discrimination following hippocampal lesions at remote time points suggests that sufficiently precise context representations for discrimination of similar contexts exist outside of the hippocampus at remote time points. However, whether these memories are identical to hippocampus-dependent memories remains to be seen. Indeed in the Wang et al (2009) study, the authors went onto do some additional tests of the hippocampal lesioned mice with intact context discrimination. When they did this they found that the spared memory was much more fragile—that discrimination disappeared with repeated testing and that extinction occurred rapidly. Therefore these hippocampal–independent memories appear to behave very differently, and are not as robust. Similar types of tests could be conducted following remote hippocampal lesions using the cocaine CPP tasks. It is likely that the hippocampus-independent memory would be similar fragile and exhibit more rapid extinction.

Another issue that might impact the dominance of hippocampus-dependent vs. hippocampus-independent traces in CPP might be the extent of training. It is possible that extensive training

protocols produce more robust memories, and these memories engage extra-hippocampal structures at earlier time points. These ‘strong training’ memories would therefore be more likely to survive hippocampal damage at earlier time-points. In contrast, weaker training protocols would produce memories that might be more vulnerable to hippocampal damage (Akers & Frankland, 2009). The current study use a relatively robust training protocol, as the sham mice still spent about 80% of total time in the cocaine-paired side at a remote time point. It would be interesting to see if future experiments could manipulate training strength and examine the sensitivity of CPP memories to hippocampus lesions at different post-training delays. Examine the process of retrieval of hippocampal and extra-hippocampal drug-associated memory traces and its interaction

Understanding how hippocampal and extra-hippocampal traces interact during memory consolidation and retrieval, especially for a remotely formed memory, is fundamental in understanding memory maturation. Since extra-hippocampal memory trace represents a more generalized contextual memory, understanding how drug-associated contextual memories generalize and what are the potential factors determining the interaction between its hippocampal and extra-hippocampal traces is critical to prevent this generalization. Several experiments have begun to characterize this interaction between hippocampal and extra-hippocampal traces. For example, in both contextual fear conditioning and CPP memories, providing reminders before remote memory retrieval makes the following memory retrieval hippocampus dependent again, suggesting that reminders facilitate the context enriched hippocampal retrieval, possibly by providing sufficient input (Debiec et al., 2002; Winocur et al., 2007). In line with this notion, it has been proposed that memory reactivation facilitates memory consolidation in cortex (Tse et al., 2007; Wang & Morris, 2010). It is also possible that hippocampus might inhibit extra-hippocampal circuits during memory retrieval, at least under some conditions. For example, Goshen et al. (2011) suggested that activation of the hippocampus may result in suppression of ACC, indicating that there might be a competition between the hippocampal and extra-hippocampal traces (Goshen et al., 2011). Future experiments are required to understand how the brain can shift between hippocampus-mediated and cortex-mediated modes of memory retrieval, and how, in the context of drug-associated memories, this would impact the expression of cocaine CPP.

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