UNIVERSITY OF CALIFORNIA SAN DIEGO

Deconstructing Context in Pavlovian Fear Conditioning

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

In

Experimental Psychology

by

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The dissertation of Kaitlin R. Van Alstyne is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

For Jim and Sam. You will always be my inspiration.

EPIGRAPH

It's more of an art. It's not an exact science.

Stephan Anagnostaras

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ABSTRACT OF THE DISSERTATION

Deconstructing Context in Pavlovian Fear Conditioning

by

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Pavlovian fear conditioning, in which a neutral stimulus is paired with a shock, has been instrumental in uncovering the neural and molecular mechanisms underlying memory since the discovery that post-training lesions of the hippocampus disrupt contextual, but not cued, fear memory. Because of the paradigm's relative simplicity, it has become the leading animal model of

declarative memory. In contrast to post-training lesions, which produce severe amnesia of contextual fear, some studies have found that mice and rats with pre-training hippocampal lesions may acquire normal contextual memory, suggesting an alternative pathway for contextual fear learning. The prevailing theory proposes that the intact hippocampus uses a configural strategy, integrating environmental cues into a cohesive representation before it can be associated with shock, while a compromised hippocampus employs an elemental strategy, associating individual cues with shock. Despite the widespread use of contextual fear conditioning, it remains unclear what animals actually learn about static "elemental" environmental cues.

This thesis introduces a novel within-subjects "context deconstruction" testing paradigm to systematically evaluate freezing responses to individual contextual components across three sensory modalities in mice. Chapter 1 reveals that intact mice exhibit an all-or-none response to the context, showing robust fear of the entire context, largely ignoring individual components, which aligns with the configural representation theory. This representation persists well into the systems consolidation period, despite repeated testing.

Chapter 2 explores the protocol's utility by identifying distinct freezing patterns to contextual components following pharmacological manipulations known to impair context memory acquisition, highlighting the sensitivity of this approach. Under some dosing regimens, particularly those that produce amnesia, mice appear to use an elemental strategy.

Chapter 3 directly tests whether hippocampal-lesioned mice acquire an elemental representation of the context. Results suggest an amalgamation of the configural and elemental hypotheses: there is evidence that mice respond to individual contextual cues, but freezing to the full training context remains more pronounced compared to freezing to partial contexts.

This thesis emphasizes the need for more thorough fear conditioning protocols to elucidate subtle deficits in context learning, contributing to advancing our understanding of learning and memory processes in normal and compromised brain states.

INTRODUCTION

Memories lay the foundation for the human experience, and how one remembers is a complex and multifaceted process. The mammalian brain remembers in a reconstructive fashion supported by the dynamic interplay of several specialized circuits. When one of these circuits is damaged, scientists are granted the opportunity to link brain and behavior, pinpointing the nuanced aspects of memory regulated by the damaged circuit.

For decades, the multiple memory systems theory (MMST) has served as the prevailing perspective on neural mechanisms underlying learning and memory. This theory posits that distinct neural circuits are responsible for different types of learning and memory processes (Squire, 1992). The case of Patient Henry Molaison (HM) was instrumental in developing this view. After bilateral surgical removal of most of his medial temporal lobe, H.M. lost the ability to form long-term ‘declarative’ memories while retaining long-term 'non-declarative' memory functions (Scoville and Milner, 1957).

Declarative memories include those consciously recalled and include memories for facts (semantic memory) and events (episodic memory). Nondeclarative memories lack the requirement of conscious retrieval and include habit and skill learning, priming, and most forms of associative learning, such as classical conditioning (Squire, 1992; Clark and Squire, 1998). Pavlovian fear conditioning, for instance, is a form of associative learning which has been extensively used as a model of emotional learning and memory in both humans

and animals (Fendt and Fanselow, 1999; Anagnostaras et al., 1999; LeDoux, 2000; Maren, 2001). One of the first and most important descriptions of fear conditioning came from Watson and Rayner (1920). They presented a young child called "Little Albert" with a white rabbit and paired that presentation with a loud clanging noise, a stimulus known to naturally evoke a fear response. After the rabbit and the loud noise were presented in a paired fashion several times, Albert showed signs of fear and distress when presented with just the rabbit alone. This outcome offered a strong example of humans learning a fear association through classical conditioning procedures.

Most people have likely been introduced to the concept of classical (otherwise called Pavlovian) conditioning through the renowned experiments of Ivan Pavlov. In his classic experiments, Pavlov introduced an initially neutral discrete conditional stimulus (CS, e.g. a metronome) to the dogs, which was presented just prior to the unconditional stimulus (US; food). After repeated pairings, the dogs formed an association between the metronome and the food, and eventually, the metronome took on a meaning of its own. Once this association between the metronome (CS) and food (US) became well established, the metronome became a conditioned stimulus (CS) of its own, eliciting the dogs' salivatory response (conditioned response; CR) without the presence of the food.

Modern laboratories conduct fear conditioning experiments in much the same way. A discrete stimulus, typically a pure tone, is presented along with an

aversive unconditioned stimulus, like an electric footshock. Once the two stimuli become sufficiently associated, the tone presented alone will elicit a fear response. A quick literature search of "Pavlovian fear conditioning" will reveal the continued popularity of the paradigm, with well over 3,000 papers returned from this search limited the last two years. One reason fear conditioning has retained its popularity over the years is that its underlying circuitry is well-defined.

Fear responses are ubiquitous throughout the animal kingdom, as they mediate defensive behaviors essential to survival (Bolles, 1970). The inability to defend oneself is more directly consequential to that organism's reproductive fitness than the failure to obtain any primary reinforcer, be it food, water, sleep, or sex. Given the magnitude of importance fear serves as a biological function, the mammalian brain has evolved an ultra-efficient and clear-cut fear conditioning circuit. Long-lasting and stable fear conditioning can occur following just one trial of learning (Fanselow, 1990; Gale et al., 2004). It is relatively simple to identify the circuitry responsible for processing a simple discrete CS and the US (with parameters for both under tight control in the experimental setup) and the point at which the circuits converge to form the basis for association.

However, in addition to discrete cues, there are also multiple static, low contingency, polymodal contextual cues making up the physical environment that are constantly present throughout the conditioning trial. This relatively more complex set of cues, collectively known as the conditioning context,

complicates the otherwise straightforward fear conditioning circuit. Discrete and contextual CSs both yield single-trial long-term fear conditioning (Mahoney and Ayres, 1976; Gale et al., 2004). They also share a point of convergence. The amygdala has long been associated with emotion and fear conditioning, and a functional amygdala is necessary for Pavlovian fear conditioning to any cue, whether discrete or contextual (Blanchard and Blanchard, 1972; Russo et al., 1976; Phillips and LeDoux, 1992; Fanselow, 1994; Maren and Fanselow, 1995; Maren et al., 1996; Maren, 2001; Pape and Pare, 2010; Fendt and Fanselow, 2013; Duvarci and Paré, 2014). Specifically, the basolateral complex of the amygdala has been implicated as a critical sensory junction for USs and CSs of both discrete and contextual nature (Herry and Johansen, 2014). However, discrete and contextual cues appear to differ in terms of the neural substrates that subserve their initial processing.

Another component of the MMST, informed by lesion studies like those of patient HM, is the identification of specific brain regions responsible for the acquisition, storage, and retrieval of information specific to a given memory type. For instance, the hippocampus has been linked to spatial and episodic memory (declarative), and the striatum has been associated with skill and habit learning (non-declarative; Squire et al., 1993; Maren et al., 1997; Logue et al., 1997). Contextual conditioning, but not discrete cue (e.g., tone) conditioning, requires the hippocampus (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). Furthermore, damage to projections from the hippocampus to the basolateral

amygdaloid complex weakens context but not tone conditioning (Maren and Fanselow, 1995). Likewise, projections from the entorhinal cortex (the gateway of information to and from the hippocampus) to the basolateral amygdaloid complex mediate contextual memory (Sparta et al., 2014). Thus, communication between the hippocampus and amygdala appears to be important for acquiring contextual fear conditioning. As such, fear conditioning has become an attractive model for studying memory, serving as an elegant example of recruitment from multiple memory systems within the same paradigm. Contextual fear conditioning rose in popularity as a model of hippocampus-dependent memory, likened to declarative-type or episodic memory (Maren et al., 2013).

Another important difference between discrete and contextual cues relates to the optimal timing of cue presentation with respect to the US. With discrete CSs, the best conditioning occurs when the CS precedes the US (i.e., forward delay conditioning). The effectiveness of conditioning and strength of the association between the CS and US improve as the delay between CS and US onset decreases, up to an optimal point. This pattern holds across various conditioning types, although there are differences in optimal timing (Schneiderman and Gormezano, 1964; Garcia et al., 1966; Mahoney and Ayres, 1976). While this timing pattern applies to various discrete CSs, it doesn't fit when it comes to contextual conditioning.

Fanselow (1986) has proposed that because contexts consist of several individual stimulus elements, the animal must learn to treat the complex stimuli as a whole unit before that unit can become a CS that is ready to associate with the US (Fanselow, 1986). This idea stems from the work of O'Keefe and Nadel (1978), who posited that a 'cognitive map' (or some form of contextual representation [Nadel and Willner, 1980, Sutherland and Rudy, 1989]) must be acquired before the shock can become associated with the context. This is not the case for a discrete stimulus, like a tone, to become a CS, as discrete stimuli are very rapidly perceived and ready to be associated with the US. This discrepancy highlights a fundamental difference in how contextual cues are processed compared to discrete stimuli in Pavlovian fear conditioning processes.

If an animal is placed into the fear conditioning context and the footshock follows immediately after, the animal will not show learning on a later context memory retrieval test, a phenomenon known as the immediate shock deficit (ISD; Fanselow, 1986). When confronted with a novel context, an animal can overcome the ISD in two main ways. It can overcome the ISD with an increased placement-to-shock interval, giving the animal ample time to explore the new environment prior to its experience of the footshock US. A baseline exploration period of around two to three minutes is ideal to reliably detect a robust contextual learning in rats or mice (Bevins and Ayres, 1995; Wiltgen et al., 2001).

The second way in which an animal can overcome the ISD is through pre-exposure to the training context at least 24 hours in advance (Fanselow, 1986, 1990; Anagnostaras et al., 2001; Rudy et al., 2004; Rudy & Wright-Hardesty, 2005; Wiltgen et al., 2006; McHugh and Tonegawa, 2007). Critically, pre-exposure to the training context permits learning in an immediate-shock protocol only if all elements of the training context are presented together; animals separately pre-exposed to individual elements of the training context continued to demonstrate the ISD (Rudy and O'Reilly, 1999). However, this finding does not necessarily suggest that animals cannot benefit from the formation of associations between the US and individual contextual cues during training. The idea that pre-exposure to the training context supports later learning highlights another difference in contextual versus discrete cues.

For discrete cues, pre-exposure causes latent inhibition, which is a Pavlovian conditioning phenomenon in which prior repeated exposure and familiarity with a stimulus prevent future associations with that stimulus from forming (Lubow and Moore, 1959; Lubow, 1989). In other words, an animal will have trouble conditioning to a cue if it has prior experience with it under neutral circumstances. This is not the case with contextual cues, as evidenced by the fact that pre-exposure and an increased placement-to-shock interval not only facilitate contextual learning but serve as a requirement for learning to occur. However, it is worth noting that the placement-to-shock interval effects on fear conditioning operate in an inverse-U shaped fashion, as fear conditioning peaks

with an interval of about 2-3 minutes and begins to attenuate significantly at about the 10-12 minutes (Bevins and Ayres, 1995; Wiltgen et al., 2001).

While fear conditioning inherently adds emotional valence to a cue, forming a representation of the context is accomplished before emotion is attached (Matus-Amat et al., 2004). In simple terms, the foundational components of fear conditioning can be broken down into the following: 1) the animal acquires a representation of the CS, 2) the animal acquires a representation of the US, and 3) the animal forms an association between the CS and US, and 4) upon re-exposure to the CS, the animal is reminded of or predicts the US, and therefore exhibits a defensive fear response to mitigate the effects of the expected US. The specific role the hippocampus plays is thought to be in assembling the contextual representation into a unified configuration of individual elements, rendering this configural unit ready and available to become associated with the US.

If the multiple polymodal components of the context must be linked together as a unified representation, the neural substrate responsible should be primed to configure. Indeed, the hippocampus possesses the unique ability to condense information from multiple sensory inputs (Suzuki and Amaral, 2004), and it is able to do so quickly. The hippocampus also plays a role in the rapid activation of plasticity-associated gene transcription, providing a mechanism for encoding one-trial experiences (Miyashita et al., 2009), which likely contributes to the formation and stabilization of contextual representations.

Some hippocampal pyramidal neurons, known as place cells, respond selectively when an animal is at a specific location providing a neuronal representation of the spatial environment (O'Keefe and Dostrovsky, 1971). The relationship of the firing patterns among these place cells is thought to enable animals to discriminate between contexts (Leutgeb et al., 2006). To become stable, hippocampal place fields require a period of exploration time, similar to the time needed to overcome the ISD in contextual fear conditioning (Frank et al., 2006). Furthermore, just as conditioning occurs more quickly in a pre-exposed context, place fields stabilize at a faster rate when animals are presented with a familiar environment (Frank et al., 2006), suggesting a temporal correspondence between the pattern of hippocampal place cell firing and contextual fear conditioning.

The discovery of grid cells in the rat entorhinal cortex (McNaughton et al., 2006) provided further evidence for a 'cognitive map' within the hippocampal formation. The firing pattern of these cells provides a coordinate system to represent an animal's position in space irrespective of landmarks, further providing neural scaffolding for cognitive spatial representations. Research in humans echoes these findings.

Clark and Squire (2013) highlight that the basic anatomy of the hippocampus is highly conserved across mammals, supporting the idea of a common "cognitive mapping" function. Hippocampal local field potentials (LFP) have also been shown to code spatial location (Agarwal et al., 2014).

Intracranial human recordings have revealed that hippocampal cells respond to specific spatial locations while navigating a virtual environment (Ekstrom et al., 2003). Machine learning methods applied to BOLD-fMRI data have demonstrated the possibility of decoding environment-specific representations within a virtual environment from patterns of hippocampal activity (Sulpizio et al., 2014). Also using fMRI, researchers have obtained evidence for grid-like representations in the human entorhinal cortex, akin to rodent grid cells, during both imagined and active (virtual) navigation (Doeller et al., 2010). Morgan et al. (2011) also used fMRI to measure human hippocampal activity, but did so while participants viewed images of their college campus and found that hippocampal activity scaled with real-world distance. Thus, the hippocampus encoded metric information in a map-like manner, even without active navigation. during imagined and active (through virtual reality) navigation.

MMST suggests that the circuits underlying a specific form of learning should be essential, and damage to an essential circuit before or after training should have similar effects. In other words, anterograde amnesia and retrograde amnesia should be proportional (Squire and Alvarez, 1995). Early dorsal hippocampal lesion studies seemed to align with this concept, suggesting the dorsal hippocampus would be the essential circuit subserving contextual conditioning.

Several studies found evidence of a moderate anterograde amnesia when dorsal hippocampal lesions were made before conditioning (Phillips and

LeDoux, 1992; Kim et al., 1993; Young et al., 1994; Maren et al., 1998). Likewise, a series of studies found evidence for retrograde amnesia when lesions were made post-training. Kim and Fanselow (1992) demonstrated that electrolytic lesions of the dorsal hippocampus (DH) made one day after training caused severe deficits in contextual fear, while sparing tone freezing. However, lesions made 28 days post-training had minimal impact, suggesting a time-limited role for the hippocampus in contextual fear memory. Maren et al. (1997) extended these findings using excitotoxic NMDA lesions, showing that while 1-day-old memories were severely impaired, 100-day-old memories were more resistant to hippocampal damage. Anagnostaras et al. (1999) further confirmed this temporal gradient within subjects, with DH lesions severely impacting recent (1-day-old) but not remote (50-day-old) contextual fear memories.

These observations of temporally-graded retrograde amnesia aligned with the earlier reports of amnesic patients, including HM, whose early-life memories were preserved despite losing memories for events for more recent memories relative to his surgery. This result has been explained as a function of cortical consolidation; over time, the initially essential role of the hippocampus in storing contextual representations is expected to be reassigned to cortical networks (Scoville and Milner, 1957; Zola-Morgan and Squire, 1990; Squire, 1992; Squire and Alvarez, 1995).

However, confusion arose after a series of experiments found little to no evidence of anterograde amnesia following NMDA (Maren et al., 1997),

electrolytic (Frankland et al., 1998), and ibotenic acid (Gerlai, 1998; Maren et al., 1998; Cho et al., 1999) lesions of the dorsal hippocampus. One reason for the mixed results obtained from these pre-training lesions appears to be due to differing training parameters. Deficits were observed when relatively weak (few, low-intensity shocks) training was deployed, but this deficit appeared to disappear under more intense training parameters (Wiltgen et al., 2006).

To account for the discrepancy in the anterograde and retrograde findings for dorsal hippocampal lesions, researchers have posited that two possible circuits exist to process context: 1) a configural process (for which evidence has been presented above), and 2) a feature-based or 'elemental' process (Winocur, 1997; Anagnostaras et al., 2001; Rudy et al., 2004; Harris, 2006; Iordanova et al., 2009; Rudy, 2009). The configural representation, which is thought to depend on the hippocampus, is processed through the integration of individual contextual cues into a unified configure. On the other hand, the elemental representation is achieved by separately associating the individual contextual elements with the shock, and this can be accomplished without a functioning hippocampus (Fanselow, 1980, 1990; Young et al., 1994; Maren et al., 1998; Anagnostaras et al., 2001). An elemental representation of the context is purportedly supported by the medial prefrontal cortex (Frankland et al., 2001; Frankland and Bontempi, 2005; Rudy, 2009; Honey et al., 2014) which, like the hippocampus, has direct projections into the amygdala and can therefore influence the expression of conditioned fear responses (Maren et al., 2013).

Recent human studies using functional magnetic resonance imaging (fMRI) further support these findings in animals, as researchers found stronger hippocampal activation for a configural representation of the context and amygdala activation for an elemental representation (Lang et al., 2009; Hermann et al., 2016; Stout et al., 2018, 2019; de Voogd, 2020; Siehl et al., 2023).

Both representations may be available, but one appears to dominate. For example, if asked to visualize a first encounter with a new campsite, one might picture the individual elements that make up the site. These might include a tent, large pine trees, a nearby rushing river, and an old canoe, but at the same time, it's conceivable to have envisioned the overarching representation of the campsite: the entire layout and atmosphere as a complete environment. Both of these representations can be associated with potential dangers (i.e., a bear) to help predict and avoid them. For instance, one might associate a tree the bear emerged from behind (an element) with the risk of being attacked, while also associating the overall campsite (the configuration) with the possibility of encountering dangerous wildlife. This dual representation of the campsite (i.e., a context), has been proposed by the dual-representation view (Rudy et al., 2004; Harris, 2006; Rudy, 2009). The theories that encompass this view propose that a context can be represented in two different ways: as a representation of individual objects (feature-based or elemental) or as a whole (configural). Both representations can become associated with a US, but the associations are organized in a hierarchical manner, as the two representations compete.

The competition between configural and elemental circuits can be explained by concepts from traditional learning theories. Various potential circuits compete during new learning, and the most efficient circuits are afforded control over memory formation and all relevant information that requires processing (Poldrack et al., 2001; Poldrack & Packard, 2003). However, alternate circuits can compensate for damage to the dominant circuit, albeit through a less efficient mechanism. This is why weaker memory performance is observed with pre-training lesions if the task is relatively difficult (i.e., remembering an event after weak conditioning). Post-training lesions to the dominant circuit would therefore be devastating because, when this circuit is strengthened, learning in the alternative circuit is hindered. Indeed, it has been proposed that the hippocampus plays two independent roles in contextual fear conditioning: (a) it stores the conjunctive representation, and (b) its outputs inhibit the ability of feature representations to become associated with the shock (Rudy et al., 2004).

Traditional learning theories have established that stimuli capable of acquiring conditioned responses contend for associative strength (Wagner et al., 1968; Rescorla and Wagner, 1972; Bolles and Fanselow, 1980; Fanselow, 1998). If two CSs are presented simultaneously, but one CS is more salient, the weaker CS will be ignored and won't be learned (Rescorla and Wagner, 1972). This phenomenon, called overshadowing (Pavlov, 1949), occurs because salient stimuli condition more readily. Likewise, when two CSs are presenting during

training, but one is more predictive of than the other, the less predictive one will be ignored, even when it might readily condition if presented alone (Wagner et al., 1968; Rescorla and Wagner, 1972). When considering how this applies to contextual versus elemental learning, one might consider the unified contextual CS as more salient and more predictive than the background elements.

As previously noted, relatively weak contextual conditioning, which is normally sufficient to produce a robust fear response in intact animals, yields a reduced fear response in those with dorsal hippocampal lesions (Wiltgen et al., 2006). As such, the proposed hippocampus-dependent configural representation appears not only to be more detailed but also processed more efficiently. If applying learning theory principles to speculate about the content of contextual learning, one can imagine that a rapidly acquired context configure (via the hippocampus) would serve as a relatively effective predictor of the US and absorb the majority of the associative strength. This is in contrast to a less efficient elemental circuit, which would require the recruitment of multiple cortical areas to establish numerous individual associations between contextual elements and the US, resulting in a slower and less cohesive learning process. Given that the more salient configure would predict the US before the less efficiently learned individual contextual elements acquire much associative strength, the elements wouldn't be afforded a chance to condition well. This prediction appears to be correct, as I observed little conditioning to the elements of context, even when contextual learning was robust (Chapter 1).

While there is a preponderance of evidence to suggest that a configural representation dominates over an elemental representation in intact animals, it remains unclear how this directly translates to what animals learn about the context in Pavlovian fear conditioning. For instance, it is unclear whether individual contextual cues of a particular sensory modality (i.e., visuospatial, tactile, olfactory) serve as relatively more salient conditional stimuli compared to others. Recently, some researchers have attempted to overcome the problem of understanding the content learned in context conditioning by looking at more behaviors, aside from just freezing (Chu et al., 2019; Borkar and Fadok, 2021; Trott et al., 2022).

However, it is essential to first understand the most commonly measured behavior before moving on to tackling a full suite of behaviors, especially when the relationship, order, and scaling between various defensive behaviors are debatable (Bolles, 1970; Fanselow & Lester, 1988; Hoffman et al., 2022).

Understanding the content of learning allows researchers to isolate the engram, or the chemical/neural trace of a learned association. If, for example, the grid stimulus (the substrate through which the shock is transmitted) soaked up the majority of conditioning, this finding would warrant an alteration to the standard contextual fear conditioning setup. The greatest weakness of contextual fear conditioning is that the context cannot be metrically varied and presented in the same way that discrete cues can be. Refining the contextual

presentation to its most important components could potentially increase the internal validity of the protocol.

Another weakness to the standard fear conditioning protocol is that the testing procedures used are simple, yielding only a few measures. Simplicity can often be favored, particularly when a protocol is used for a purpose that necessitates efficiency, like drug screening for cognitive effects (Anagnostaras et al., 2000; Anagnostaras et al., 2010). However, the testing regimen of fear conditioning is generally pretty sparse and uses many animals to obtain a relatively small amount of data. Often an enitre fear conditioning experiment lasts only three sessions, and the animals are not reused, because it is assumed that repeated testing would harm the measures and produce extinction, even though fear conditioning is fairly resistant to extinction.

To address these weaknesses, this dissertation work presents and offers support for the utility of a novel and intensive 'contextual deconstruction' testing procedure that allows the content of contextual fear to be tested with higher precision without the use of additional animals. In Chapter 1, the new protocol is first characterized in intact mice under two different training protocols (signaled 'background' or tone present conditioning, and unsignaled 'foreground' or tone absent conditioning) purported to differentially favor an elemental over a configural contextual representation (Phillips and LeDoux, 1994). All mice, across both training conditions, were all retested using the 'contextual deconstruction' protocol 50 days after their original training to determine the stability of the

contextual memory representation over time. Overall, the results of Chapter 1 revealed two main findings: (1) Despite having 10 testing days, mouse contextual fear responses remained stable, with little extinction and minor forgetting, suggesting considerable opportunity for within-subjects testing is being missed in the standard paradigm. (2) Results revealed very strong evidence that mice learn the context as a whole and show very little fear to contextual elements. This was true regardless of whether elements were combined in pairs, if signaled ('background') or unsignaled (foreground') conditioning is used, or if the memory is recalled recently or remotely with respect to the original training event, although there was some evidence that unsignaled contextual learning became somewhat more elemental with age. Together, these data suggests that intact mice strongly favor a configural representation of the context.

Chapter 2 explores the nature of contextual representations after mice are trained on a range of doses of three drugs (scopolamine, an anticholinergic, and two NMDA-receptor antagonists, CPP and esketamine) known to impede context memory acquisition. While context memory deficits attributed to these different amnesic mechanisms have previously been lumped together and termed generally as 'context amnesia,' the results of Chapter 2 experiments revealed nuanced but meaningful differences in response patterns to individual contextual cues depending on the unique pharmacological agent that was used to induce a memory deficit. Interestingly, mice trained on scopolamine

and esketamine appeared to represent the context in an elemental fashion, even at low doses that did not produce a noticeable amnesic effect. Mice trained on CPP continued to represent the context in a strictly configural manner, even at a dose with a minor amnesic effect (5 mg/kg). However, mice trained at a dose producing a robust context fear memory deficit (10 mg/kg) appeared to learn about the context elementally.

The experiment of Chapter 3 further develops this protocol by directly testing the standard model of the hippocampus' role in contextual fear memory. Traditional data analyses focusing on mean differences between experimental groups (i.e., lesions versus controls) revealed no major differences. However, more sensitive measures capable of detecting nuanced response patterns between experimental groups revealed a shift toward an elemental representation in the lesioned mice. The context deconstruction protocol provided a wealth of within-subjects data not afforded by the standard protocol, granting us the opportunity to use a more powerful and sensitive data analysis method to detect nuanced change in contextual representation.

Together, the results of this body of work offer strong evidence of the 'contextual deconstruction' protocol's utility. It offers an efficient means to collect much more data using the same number of subjects, and it is sensitive enough to reveal previously masked nuanced changes to contextual representations following a variety of manipulations to learning and memory processes.

CHAPTER ONE

Putting the pieces together: The content of contextual learning

In recent years, Pavlovian fear conditioning has emerged as a leading model by which to study memory, as it can evaluate hippocampus-dependent memory with a strong degree of temporal precision and relatively low effort (Anagnostaras et al., 2000; Anagnostaras et al., 2010). In the standard understanding of the task, CS-US associations are formed in the amygdala. The role of the hippocampus is thought to be to form a representation of the context CS through a configural, spatial, or similar learning process (Wickelgren, 1979; Sutherland & Rudy, 1989; Fanselow, 1984; Kim & Fanselow, 1992; Anagnostaras et al., 2001).

Despite the elegance of the standard model, numerous confusing findings exist regarding the role of the hippocampus in contextual fear. For example, if hippocampal lesions are made immediately post-training, a robust deficit is observed, whereas if they are made pre-training, a less substantial, sometimes nonexistent deficit is observed (Maren et al., 1997; Maren et al., 1998; Frankland et al., 1998). A hypothesis has been offered suggesting that contextual fear can be acquired in two ways: through a unified or configural/spatial representation or through an elemental representation, whereby individual elements which comprise the context are each weakly associated with the shock and then summate to produce significant fear (Frankland et al., 1998; Anagnostaras et al., 2001). It is argued that with an intact hippocampus, the unified strategy

dominates, because the context has better predictive value and higher salience than the individual cues (Anagnostaras et al., 2001). However, in animals with hippocampal damage, the elemental strategy is the only one available. Implicit in this argument is that intact animals do not learn to fear the static elemental cues, although this has still not been directly tested (Holland & Bouton, 1999).

There is an abundance of indirect evidence to suggest that Intact animals favor the configural process. As previously discussed, contexts require a prolonged exposure period to support acquisition, arguably due to the time needed to cognitively assemble the contextual representation, which may be represented as something like a 'cognitive map' (O'Keefe and Nadel, 1978; Nadel and Willner, 1980; Sutherland and Rudy, 1989). The preference for configural over elemental processing in intact animals is also supported by the finding that animals with dorsal hippocampal lesions have difficulty discriminating between similar contexts, even when they appear to have no deficit in conditioning to the training context (Frankland et al., 1998). This suggests that a configural representation, supported by a functioning hippocampus provides a more stable and detailed contextual representation. Likewise, rats with hippocampal damage or inactivation demonstrate decreased performance on tasks such as the transverse-patterning problem, which requires the formation of a configural representation, but they can still

solve relatively simpler tasks of elemental discrimination (Alvarado and Rudy, 1995; Iordanova et al., 2009).

Furthermore, as previously stated, dorsal hippocampal lesions made prior to training may cause a negligible freezing deficit, if any, but post-training lesions consistently produce a profound retrograde amnesia for contextual fear memory (Maren et al., 1997; Anagnostaras et al., 2001). The dual-representation view of contextual learning would suggest that this pattern of results stem from intact animals primarily relying on a configural representation formed in the hippocampus; once lesions render the hippocampus unavailable after conditioning, that configural representation is lost. This compensation process of shifting from configural to elemental contextual learning must take some time to develop, as direct injections of various agents such lidocaine, the NMDA receptor antagonist AP5, or scopolamine just before conditioning still produce a large impairment (Gale et al., 2001; Schenberg et al., 2005; Chang et al. 2008). In the typical lesion experiment, a two week period of recovery Is afforded (Maren et al., 1997; Anagnostaras et al., 2001).

Although a configural strategy appears to be dominant, there is evidence to suggest that contextual cue types of certain sensory modalities may be more salient to mice, and thus might acquire a stronger associative relationship to the US relative to other contextual cues. Garcia first emphasized the biological significance of cue types in his fear conditioning and taste aversion 'bright-noisy water' experiment, in which rats were presented with light and sound cues with

saccharin flavored water; following this, rats were either fear conditioned with shock or given taste aversion with lithium chloride or radiation (Garcia and Koelling, 1966). The dependent variable was avoiding licking of the water, a measure that can indicate fear or taste aversion. Surprisingly, the rats given shock showed much greater fear to the audiovisual cue, whereas those poisoned showed greater aversion to the saccharin flavor. This work demonstrated that not all stimuli are equally salient in forming associations, and animals are biologically prepared to associate certain stimuli with specific outcomes more easily than others. In mouse fear conditioning, this could mean that some contextual elements (e.g., odors or textures) might be more readily associated with fear than others (e.g., visual cues), depending on their ecological relevance to mice.

There may also be specific training conditions that facilitate an elemental strategy. Mice and rats demonstrate significantly greater freezing when tones are not paired with the shocks during conditioning ("unsignaled training"; Gerlai, 1998; Anagnostaras et al., 1999; Calandreau et al., 2005). According to this argument tones compete with the context and conditioning without tones brings the context into the foreground of the animal's attention. When the context is placed in the foreground, mice with pre-training lesions exhibited greater levels of freezing compared to those trained with the context in the background, signaled condition (Phillips & LeDoux, 1994; Gerlai, 1998). It may be

that an elemental strategy is more successful when the tone does not compete with or overshadow the context.

The acquired representation may also change over time as it is consolidated; for example, one prominent model of systems consolidation posits that memories change from episodic to semantic as they consolidate from the hippocampus to cortex (McClelland et al, 1995; Frankland et al., 2001; Frankland and Bontempi, 2005). It has been suggested that contextual memory could therefore evolve from configural to elemental across the consolidation period (e.g. Pause et al., 2013). In some studies, hippocampus-dependent memories naturally lose both their precision and strength with strong generalization developing between contexts over time (Wiltgen and Silva, 2007; see also Winocur et al., 2007; Ruediger et al., 2011; Jasnow et al., 2017). Fifteen days after training, Biedenkapp & Rudy (2007) found that rats froze to comparable levels for the training context and an altered context containing a subset of the original training context features. As these animals responded strongly to a subset of features, this may be because certain contextual elements become more important than others over time. Wiltgen et al. (2010) note that generalization over time is probably a reflection of a shift in the responsibility of memory storage from the hippocampus to the cortex during long-term consolidation (Frankland et al., 2001; Frankland and Bontempi, 2005). This shift in responsibility can explain why fear memory is disrupted when hippocampal lesions are made one day after training, but memory remains intact when

lesions are made weeks after training (Frankland et al., 1999; Anagnostaras, Maren, & Fanselow, 1999). It is possible that some contextual cues are more salient than others and are consequently more readily retrieved after memories are consolidated long-term.

Although there is much support for a dominant configural strategy in the literature, it is yet undetermined what types of context stimuli or training types might favor a configural strategy over an elemental strategy in intact animals (Holland & Bouton, 1999; Huckleberry et al., 2016). Thus, we systematically manipulated the similarity of individual contextual cues relative to the training context during post-training transfer tests to assess whether certain cues of various sensory modalities (odor, visuo-spatial, tactile) may support an elemental contextual learning. In addition, we manipulated the conditioning protocol (signaled tone-shock pairings versus unsignaled shocks) to determine whether having the context in the "foreground" or the "background" affects which mnemonic strategy will be used. Rudy (2009) found that hippocampal dominance in contextual fear conditioning may be overcome with multiple training sessions. Thus, the present study utilized three tone-shock pairings during our signaled training protocol to thoroughly condition the animal and promote conditions for elemental learning. Lastly, the present study tested whether contextual and elemental learning generalized over time.

To address these questions, we conducted two experiments, all using the Pavlovian fear conditioning paradigm. In the first experiment, mice were trained

with three tone-shock pairings (Signaled), placing the context into the background. Beginning the day after training, the mice completed ten Recent Memory post-training context transfer tests, in a modified counterbalanced fashion, evaluating the relative contribution of different aspects of the context to contextual fear. Two months later, the same mice were retested without any additional training (Remote Memory). For the second experiment, we repeated the first experiment (Signaled/Recent) without the tone, but using two unsignaled shocks to equate the initial level of conditioning, as Unsignaled training produces greater fear.

Materials and Methods

Animals

Twenty hybrid C57BL/6Jx129S1/SvImJ (129B6; Jackson Laboratory, West Sacramento, CA, USA; 11 male and 9 female) mice were used for Experiment 1 (signaled training), and twenty-four (12 male and 12 female) mice were used for Experiment 2 (unsignaled training). This mouse strain was selected based on recommendations made at the Banbury Conference on Genetic Background in Mice, which was held to facilitate the comparison of results between experiments and among laboratories. This strain is often selected because of its strong performance on behavioral memory measures, and its relevance to standard stem cell lines along with standard breeding practices in the generation of mutant mice (Silva et al. 1997). Mice were weaned at 3 weeks of age and group-housed (2–5 mice per same-sex cage) with unrestricted access

to food and water. The animal colony was maintained on a 14:10-h light/dark schedule, and all experimental procedures occurred during the light phase. Mice were at least 10 weeks old and handled for 3 days (1 min/day) prior to fear conditioning and testing. All animal care and experimental procedures were approved by the UCSD IACUC and compliant with the NRC Guide for the Care and Use of Laboratory Animals.

Fear Conditioning

The well-validated VideoFreeze software (Med-Associates Inc., Georgia, VT, USA) captured and scored each individual mouse's freezing behavior from an infrared camera (Anagnostaras et al. 2000, 2010). This program controlled the tones and shocks to the chambers. Up to seven mice were trained and tested simultaneously in individual fear conditioning chambers (32 x 25 x 25 cm), which consisted of stainless-steel sidewalls, white acrylic back walls, and clear polycarbonate front and top walls. Each chamber was transformed across three sensory dimensions to create distinct contexts (cues described below). Invisible 980 nm near-infrared (NIR) light illuminated the chamber for the camera, which had a visible light filter so that visible lighting could be altered without affecting computer vision and scoring. Likewise, internal components of the chamber used infrared transmitting acrylic that appeared black in visible light, but were transparent to NIR light (Anagnostaras et al. 2010).

Context Cues

For all mice, in both *Experiment 1* and *Experiment 2,* all contexts consisted of three cues of three different sensory modalities (olfactory, visuo-spatial, and tactile). The full training context consisted of a strong 50% ethanol odor (olfactory), a white house light (~80 lux) and black triangular teepee (visuo-spatial), and a stainless steel grid floor (tactile). The neutral context consisted of no odor (boxes were cleaned with 7% isopropanol, then rinsed with water), no visible light or teepee (visuo-spatial), and a white acrylic floor (tactile). Fig. 1.1 includes a depiction of both training and neutral contexts. Examples of the training and neutral contexts are depicted in Fig X. Prior to placing a mouse into the chamber, all surfaces within the space were cleaned with an odor matching the intended olfactory cue: either 50% ethanol or water (cleaning with 7% isopropanol rinsed with water to minimize any residual odor) and wiped dry. Extra care was taken to ensure the strong ethanol odor did not linger when a chamber presentation was intended to represent the absence of the olfactory cue.

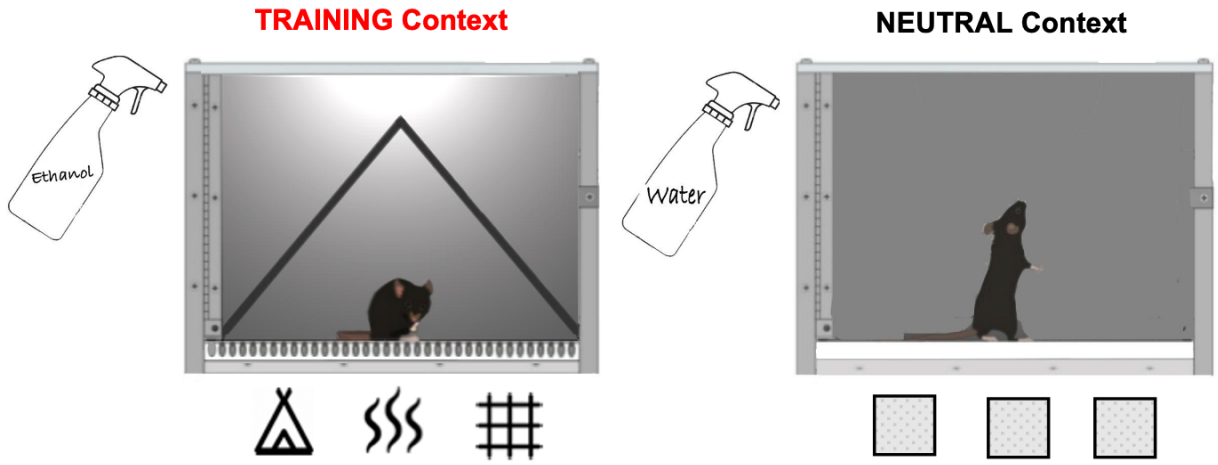

Figure 1.1: Diagram of training and neutral contexts. The training context consisted of a strong ethanol scent (olfactory), a triangular teepee and house light (visuo-spatial), and a stainless steel grid floor (tactile). The neutral context was cleaned with a mild isopropanol mixture before being wiped clean with water (olfactory), the house light was turned off and teepee was removed (visuo-spatial), and the grid floor was covered by a piece of white, opaque acrylic (tactile).

Experimental Procedures

Fear Conditioning Training: Signaled (Experiment 1)

Mice were placed inside the chamber with all the training cues (50% ethanol odor, house light and black teepee insert, and grid floor) present. The mice were recorded for 2 minutes prior to tone-shock pairings for baseline freezing and locomotor activity measures. The mice were given this pre-shock time period to allow them to adequately explore their environment, preventing the immediate shock deficit (Fanselow, 1986, 1990; Frankland et al., 2004; Wiltgen et al., 2006; McHugh & Tonegawa, 2007). Following the baseline period, the mice experienced three tone-shock pairings. Each pairing consisted of a single 30s pure tone (2.8kHz, 85dBA) that co-terminated with a 2s scrambled foot

shock (0.75 mA, AC, RMS constant current) that was transmitted through the grid floor. The interval between pairings and after the final pairing was 30s. Thus, the mice were in the chamber for a total of 5 min during training. VideoFreeze software scored freezing behavior and movement throughout the entire session (Anagnostaras et al., 2010).

Fear Conditioning Training: Unsignaled (Experiment 2)

For this experiment, the mice were placed in the same training context as in *Experiment 1,* but they received only two shocks. No tones (unsignaled) were presented at any time in this experiment. Two shocks were delivered rather than three to avoid ceiling effects and equate the level of initial freezing behavior with Experiment 1.

Context testing: Experiment 1 and Experiment 2

Beginning the day after training, each mouse was tested on one context per day for ten consecutive days. On day one, all mice were tested on the neutral context, which is described above. On day two, all mice were tested on the original training context, also described above. The first two days consisted of baseline maximum responding to the training context and baseline minimum responding to the neutral context. On days 3-8, mice were tested on one of six partial contexts, where one or two cues matched the original training context, and the remaining cue(s) were from the neutral context. Partial contexts were counterbalanced over these six days. In order to evaluate the effects of repeated testing (for example, to determine if extinction occurred), two

additional anchoring tests were completed on days 9 and 10. On the ninth day, all mice were retested on the neutral context, and on the tenth day all mice were retested on the full original training context. For each test, mice remained in the chamber for 5 minutes without experiencing any tones or shocks. Freezing was scored for 5 minutes to measure the cumulative effect of individual contextual cues on fear memory.

Remote testing: Experiment 1 and Experiment 2

All mice in *Experiment 1* and *Experiment 2* completed a second set of contextual tests beginning 50 days after their initial training. No additional training (shocks) was administered prior to the set of remote memory tests. All ten remote memory tests followed the exact procedure as the recent context tests.

Statistical Analyses

Mean differences in freezing data for this set of experiments were analyzed using multivariate analyses of variance (MANOVAs) with one or two within-subjects factors. In each of these analyses, percent freezing served as the dependent measure. Data from male and female mice were collapsed, as no statistically significant differences were found based on sex ($ps > 0.05$). Post hoc comparisons were performed following significant group differences using Tukey's Honest Significant Difference (HSD) test. All data are presented as means ± standard error the mean (SEM). MANOVAs and post-hoc analyses were conducted in Jamovi (version 2.5), an open-source statistical analysis software.

Graphical representations of the data were created using Graphpad Prism 9 (GraphPad Software, LLC).

To compare the fit of custom configural and elemental models of contextual fear conditioning (see Fig 1.1), a permutation test focusing on the predictive accuracy for intermediate cue conditions (1 and 2 matching contextual cues) was employed. These analyses were performed using R (version 4.2; R Core Team, 2024) with the dplyr package.

For every condition (i.e., signaled recent), mean freezing responses were calculated for each cue condition (0, 1, 2, and 3 cues matching the training context) across all subjects. The configural model predicted constant freezing levels for 1 and 2 cues (equal to the observed mean for 0 cues), with a sharp increase in freezing from 2 cues to 3 cues (see Figure 1.2 for hypothetical data that ideally fit each model). The elemental model predicted a linear increase in freezing from 0 to 3 cues, with a trend line passing through the observed means for 0 cues and 3 cues.

The analyses focused on the observed and predicted values for the 1 and 2 cue conditions, as these represent the critical difference between the two models. The goodness of fit for each model was quantified using Mean Squared Error (MSE) between the observed individual data points and the predicted values.

To assess the statistical significance of the difference in fit between the two models, a permutation test with 10,000 iterations was performed. In each

iteration, the assignment of predicted values to the configural and elemental models was randomly permuted, and the difference in MSE was recalculated. The p-value was computed as the proportion of permutations yielding a difference in MSE as extreme as or more extreme than the observed difference.

For both Experiment 1 (signaled training), a total of 120 (6 freezing scores: 3 from the 1 cue group and 3 from the 2 cues group, from 20 mice) individual data points from the 1 and 2 cue conditions combined were analyzed. For Experiment 2 (unsignaled training), 144 data points (from 24 mice) were analyzed. The analysis provided MSE values for each model and a p-value indicating the statistical significance of the difference in model fits.

This approach was used to determine whether one model provided a significantly better fit to the observed data for the critical intermediate cue conditions, while taking into account the variability in individual responses.

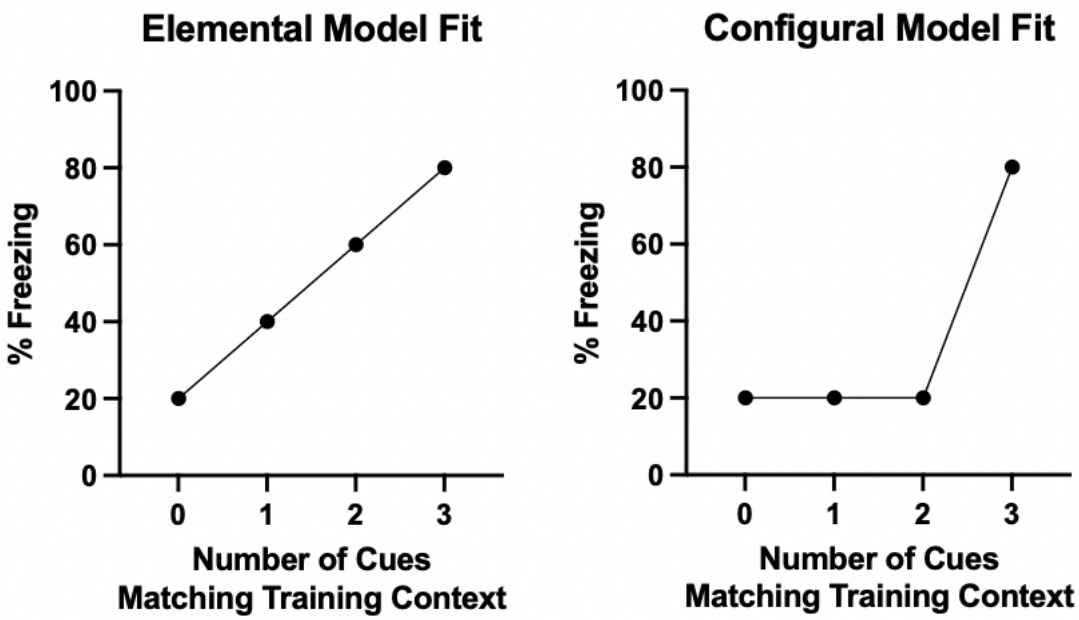

Figure 1.2: Hypothetical freezing data reflecting the ideal data trends underlying an elemental and a configural representation on a given context memory test.

Results

Experiment 1: Signaled Training

A MANOVA using two within-subjects factors (memory age x individual context test) revealed a significant main effect of individual context test type [F (9, 171) = 40.34, $p < 0.001$]. There was no significant main effect of memory age [F (1, 19) = 0.49, $p = 0.49$; see Fig 1.7]. However, there was a significant interaction between memory age and context test factors [F (9, 171) = 3.42, $p < 0.001$].

Another MANOVA with two within-subjects factors (memory age x number of training cues) revealed a significant main effect of the number of cues matching the training context [F (3, 57) = 124.69, $p < 0.001$] on freezing. Individual freezing scores were aggregated across each cue group per mouse to consolidate the freezing data, resulting in a single representative freezing score for each mouse within each cue category. For example, in the case of contexts featuring a single cue that matched the training environment, the freezing scores from the three partial contexts containing just one training cue were averaged, yielding one comprehensive score per mouse for the '1 cue' group. There was no significant main effect of memory age [F (1,19) = 0.21, $p = 0.65$; see Fig 1.8], indicating that freezing from recent to remote time points did not differ significantly overall. There was, however, a significant interaction between memory age and number of training cues [F (3,57) = 6.31, $p < 0.001$].

A final MANOVA (memory age x cue type across all six partial context tests) revealed a significant main effect of cue type [$F(5, 95) = 35.15$, $p < 0.001$] on freezing scores for the partial contexts (i.e., those with only 1 or 2 cues matching the training context). There was no main effect of memory age [$F(1,19) = 2.74$, $p = 0.11$; see Fig 1.8]. Likewise, there was no significant interaction between cue type and memory age [$F(5,95) = 1.57$, $p = 0.18$]. Individual freezing scores were aggregated across each cue type per mouse to consolidate the freezing data. This resulted in a single representative freezing score for each mouse within each cue type grouping (e.g., teepee present, teepee absent).

Post-hoc analyses: recent memory following signaled (tone-paired) training. After a 24-hour consolidation period following tone-paired conditioning, all mice began a ten-day testing regimen.

Individual Tests: partial contexts versus full training context. Post-hoc Tukey comparisons revealed that the average percent freezing scores for the first (63.1 ± 5.69) and second tests of the training context (62 ± 4.44) were both significantly higher compared to average percent freezing scores for all six partial context tests (*ps* < 0.001 for all comparisons). This indicates that no combination of two training cues yielded a freezing response as strong as the full context containing all three original cues.

Individual Tests: partial contexts versus neutral context. In addition, none of the average percent freezing scores for the six partial contexts (ranging from 18.6 to 35.3) differed significantly from average freezing to the first and second

neutral context tests (*ts* < 2.33, *ps* > 0.05). Thus, the mice appeared to respond to all six partial contexts as if they were presented with the neutral context, indicating that they did not learn about the individual cues during training.

Individual Tests: Extinction. Percent freezing scores did not differ ($t = 0.17$, $p = 1.0$) between the first and second tests of the full training context. This indicates that there was no extinction to the training context, even after several presentations of contexts with shared individual contextual cues.

Individual Tests: Generalization. Tukey comparisons also revealed that the average percent freezing score for the first test of the neutral context (23.6 ± 4.34) was not significantly different compared to percent freezing to the second test (23.3 ± 3.66) of the neutral context ($t(19) = 0.1$, $p = 0.99$); this finding indicates that no generalization to neutral stimuli occurred, despite the eight context tests that preceded the second presentation of the neutral context.

Number of Cues. When memory was assessed within a week of training, mice that underwent tone-paired training froze significantly more to the entire training context (3 cues group) compared to how they froze to contexts with 0, 1, or 2 training cues (*ts* > 11.75, *ps* < 0.001). Freezing scores across the 0, 1, and 2 cue groups were not significantly different (*ts*< 1.7, *ps* > 0.05). Together, these findings provide further evidence that intact mice learn and respond to contextual cues in an all-or-none fashion.

This data set was also used to assess the goodness of fit of contextual freezing data (after signaled training) to custom elemental and configural

models (see Table 1.1). This approach determined that the configural model provided a significantly better fit for the observed data for the critical intermediate cue conditions (1 or 2 cues matching the training context). Thus, recent contextual memory following signaled training appears to be represented in a configural manner.

Table 1.1: Goodness of Fit Comparison between custom configural and elemental models for contextual freezing data (recent memory) following signaled training. Mean squared errors (MSE), p-value, and observed vs. predicted freezing values for partial contexts with 1 and 2 cues matching the original training contexts are reported. The configural model fit the data significantly better compared to the elemental model ($p < 0.001$).

Signaled Recent	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	22.42	22.42	402.74	
Elemental Expected	34.88	47.35	658.74	
Actual Values	23.59	26.5		< 0.001

By cue type (only partial context tests). Across the six partial contexts, an analysis of freezing scores was conducted to determine the overall effect of the presence or absence of each of the three individual training cue types (visuospatial/teepee, olfactory/ethanol, and tactile/grid). Mice froze significantly more for partial contexts containing the teepee cue compared to partial contexts without the teepee [$t(19)=4.15$, $p = 0.02$]. However, the mean increase in freezing attributed to the teepee's presence was only about 10%. This is a minor increase, considering the mice froze twice as much (about 20%) to the presentation of a completely neutral/novel context. Mice did not freeze

differently depending on whether ethanol or the grid was present in the partial contexts (*ts* < 2.46, *ps* > 0.05).

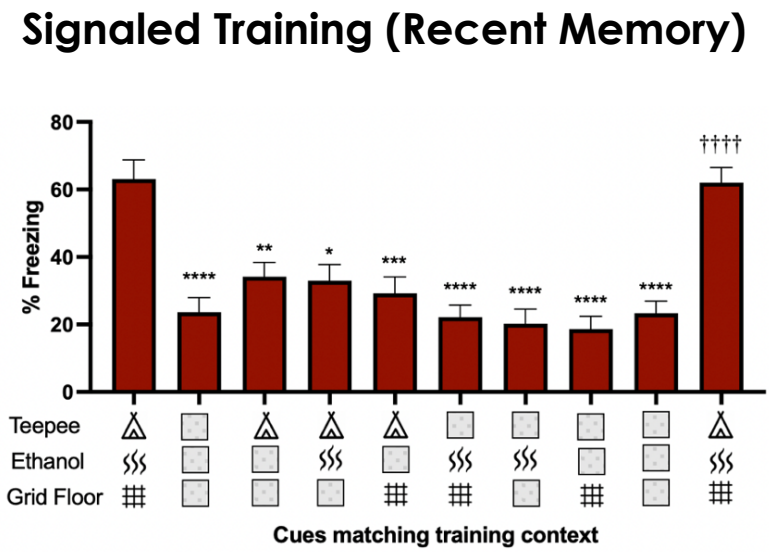

Figure 1.3: Percent freezing (a proxy for context memory) tested 1-10 days after signaled (tone-paired) fear conditioning. One context test was administered each day for ten consecutive days. This 'recent' memory is considered long-term consolidated memory, as synaptic consolidation has occurred, but the memory is not expected to have undergone complete cortical consolidation at this point. Each bar represents the mean percent freezing score for each individual context test. Symbols below each bar indicate the cues in each context configuration matching the training context; grey boxes indicate the absence of a given training cue. Asterisks (*) represent significance against the first test of training context memory. Daggers (†) represent significance against the first test of the neutral context. Error bars represent SEM. For each comparison, * or † *Ps* < 0.05, ** or †† *Ps* < 0.01, *** or ††† *Ps* < 0.001.

Signaled/Recent: Aggregated Cue Analyses

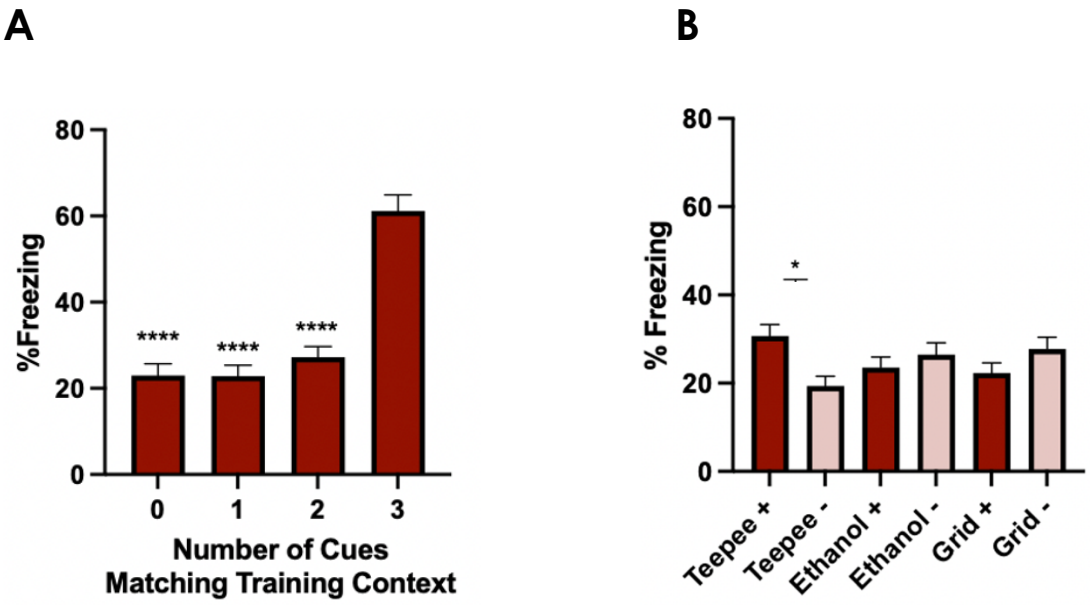

Figure 1.4: A) Aggregated mean context freezing data (from all ten context tests) based on the number of cues matching the original training context. Asterisks represent significant comparisons against the 'three cues' group. The groups with 0, 1, or 2 cues matching the training context did not differ significantly. **B)** Relative importance of cues from three sensory modalities: visuospatial, olfactory, and tactile. Aggregated mean freezing data grouped by the presence or absence of the three original training context cues (teepee/visuospatial, ethanol/olfactory, and grid/tactile). Freezing from only the six partial contexts is included. Data are depicted as means +/- SEM. Asterisks represent significant comparisons between the presence and absence groups for each cue type. For each comparison, * *Ps* < 0.05, ** *Ps* < 0.01, *** *Ps* < 0.001.

Post-hoc analyses: remote memory following signaled (tone-paired) training. Mice were retested 50 days after the initial testing to determine if generalization or any change in responsivity to the various cues was seen.

Individual Tests: partial contexts versus training context. At the remote time point, the mice froze significantly less to the partial contexts without the teepee compared to the first test of the original training context (*ts* > 6.38, *ps* <0.001). However, their freezing was not significantly different to the first training context test compared to the partial contexts containing the teepee (*ts* < 3.65, *ps* > 0.05).

Individual Tests: comparison against first neutral context test. Mice froze significantly more to two of the partial contexts containing the teepee (teepee alone and teepee + ethanol) than they did on the first test of the neutral context (*ts* >5.04, *ps* < 0.02). However, their freezing was not significantly different across all other partial contexts (including the teepee + grid context) compared to their freezing on the first neutral context test (*ts* < 3.4, *ps* > 0.05). This suggests that their memory for the context became slightly less precise over time; in particular, they had some trouble distinguishing whether the context was safe when the teepee was present.

Individual Tests: Extinction. There was no evidence of extinction, given that freezing to the first and second presentations of the training context did not differ [$t(19) = 2.92$, $p = .36$]. This is a testament to the stability of the original

contextual memory trace over an extended period of time, despite the fact that 19 context tests preceded the final training context test.

Individual Tests: Generalization. There was also no evidence of generalization to the neutral context at the remote timepoint, as freezing between the first and second neutral context tests did not differ [$t(19) = 1.02$, $p = 0.99$]. Freezing to the second presentation of the neutral context also remained significantly lower compared to freezing to the second presentation of the training context [$t(19) = 5.49$, $p < 0.003$]. This further highlights the stability of the context memory trace over time in the C57BL/6Jx129S1/SvImJ hybrid strain.

Number of Cues. The pattern of freezing at the remote timepoint largely mirrored that of the recent timepoint with respect to the number of cues matching the training context. Mice froze significantly more for the full training context (with all three cues present) compared to all other contexts (with 0, 1, or 2 cues present; $ts > 6$, $ps < 0.001$). The only difference was that there was a significant increase from the 0 cues to 2 cues groups [$t(19) = 3.66$, $p = 0.03$], likely due to minor responding to the teepee cue.

This data set was also used to assess the goodness of fit of contextual freezing data (after signaled training) to custom elemental and configural models (see Table 1.2). This approach determined that the configural model provided a significantly better fit for the observed data for the critical intermediate cue conditions (1 or 2 cues matching the training context). Thus, recent contextual memory following signaled training appears to be

represented in a configural manner, even after significant time has lapsed since the original training period.

Table 1.2: Goodness of Fit Comparison between custom configural and elemental models for contextual freezing data (remote memory) following signaled training. Mean squared errors (MSE), p-value, and observed vs. predicted freezing values for partial contexts with 1 and 2 cues matching the original training contexts are reported. The configural model fit the data significantly better compared to the elemental model ($p < 0.001$).

Signaled Remote	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	25.4	25.4	518.68	
Elemental Expected	35.22	45.04	608.91	
Actual Values	28.95	36.33		< 0.001

By cue type. Mice froze significantly more for partial contexts containing the teepee cue compared to partial contexts without the teepee [$t(19) = 7.57$, $p < 0.001$]. The mean increase in freezing attributed to the teepee's presence was about 20%, which is about equal to the average amount of freezing observed to the presentation of the neutral context at the remote timepoint. Mice did not freeze differently depending on whether ethanol or the grid was present in the partial contexts ($ts < 3.11$, $ps > 0.05$).

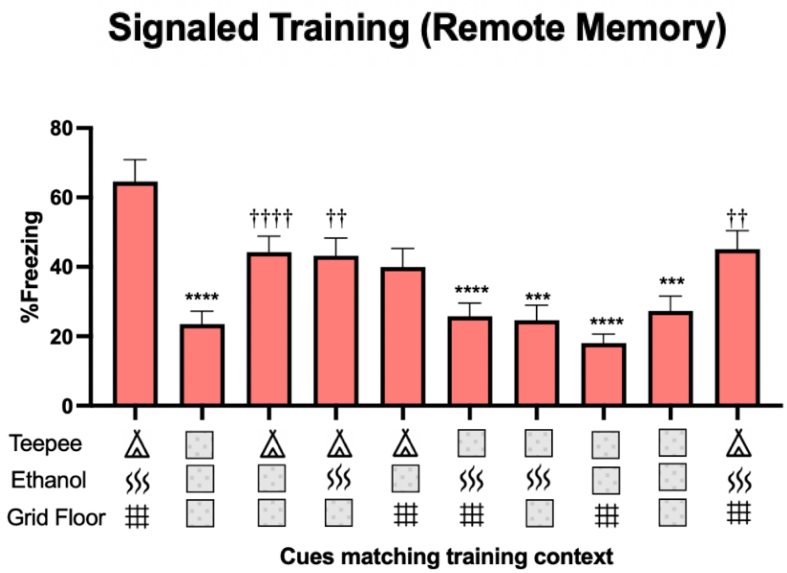

Figure 1.5: Percent freezing (a proxy for context memory) tested 50 days after signaled (tone-paired) fear conditioning. This 'remote' memory is considered long-term consolidated memory, well after cortical consolidation has occurred. One context test was administered each day for ten consecutive days. Each bar represents the mean percent freezing score for each individual context test. Symbols below each bar indicate the cues in each context configuration matching the training context; grey boxes indicate the absence of a given training cue. Asterisks (*) represent significant comparisons against the first test of training context memory. Daggers (†) represent significant comparisons against the first test of the neutral context. Error bars represent SEM. For each comparison, * or † *Ps* < 0.05, ** or †† *Ps* < 0.01, *** or ††† *Ps* < 0.001.

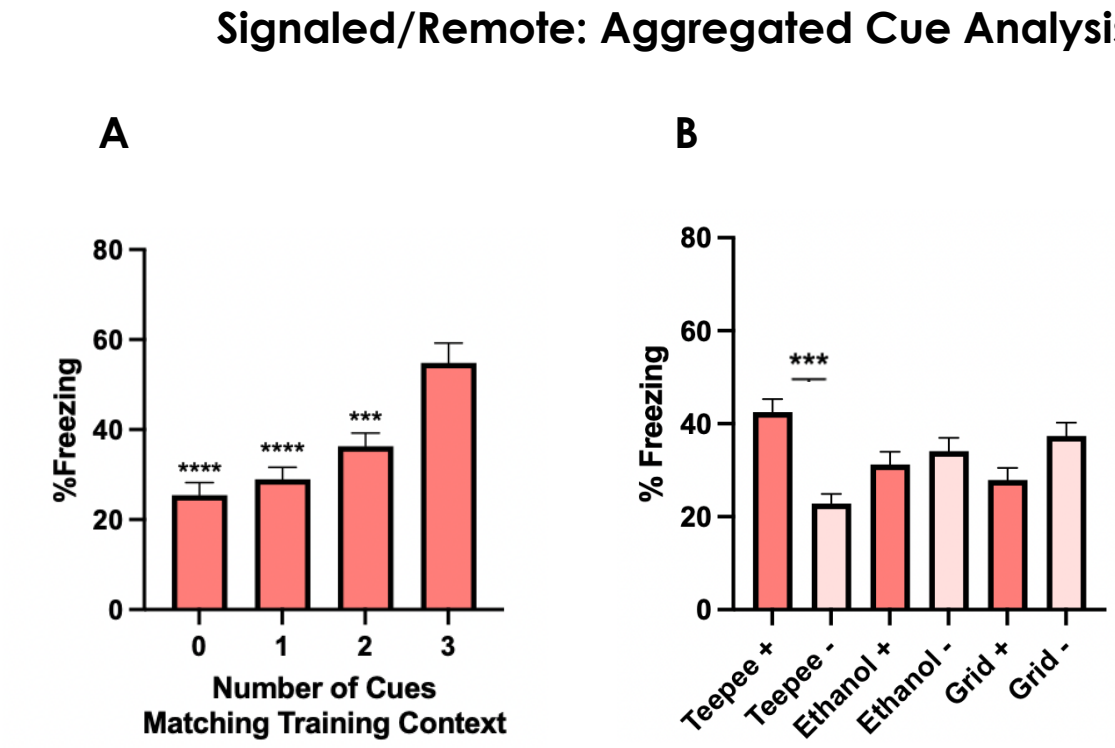

Figure 1.6: A) Aggregated mean context freezing data (from all ten context tests) based on the number of cues matching the original training context. Asterisks represent significant comparisons against the 'three cues' group. The groups with 0, 1, or 2 cues matching the training context did not differ significantly. **B)** Relative importance of cues from three sensory modalities: visuospatial, olfactory, and tactile. Aggregated mean freezing data grouped by the presence or absence of the three original training context cues (teepee/visuospatial, ethanol/olfactory, and grid/tactile). Freezing from only the six partial contexts is included. Data are depicted as means +/- SEM. Asterisks represent significant comparisons between the presence and absence groups for each cue type. For each comparison, * *Ps* < 0.05, ** *Ps* < 0.01, *** *Ps* < 0.001.

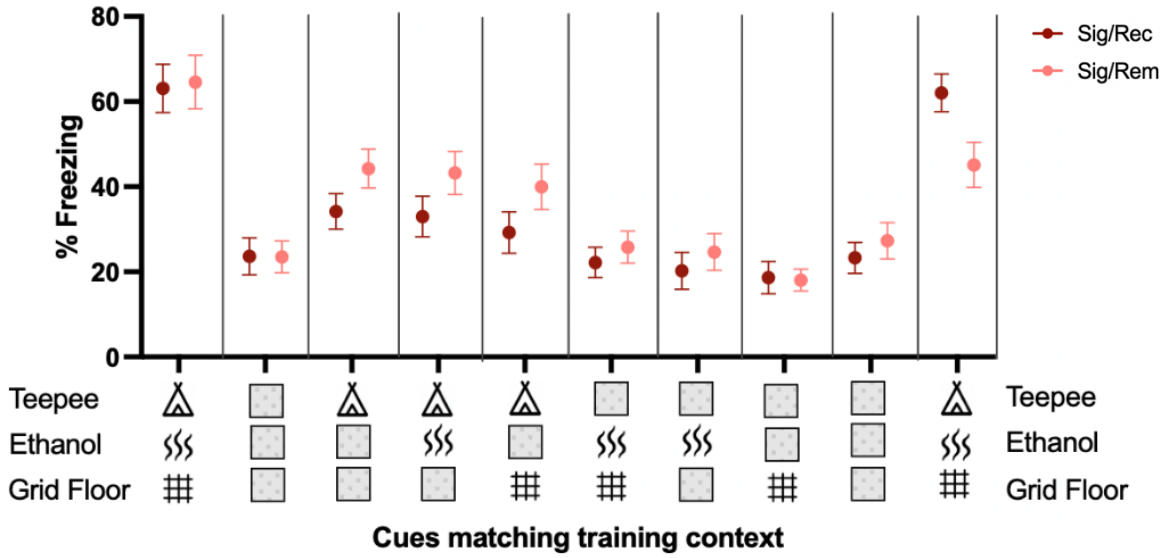

Figure 1.7: Comparison of recent and remote memory after signaled training. Maroon symbols represent recent memory (Sig/Rec) and pink symbols represent remote memory (Sig/Rem). Symbols below each set of recent and remote points indicate the cues in each context configuration matching the training context; grey boxes indicate the absence of a given training cue. Error bars represent SEM. There were no significant differences depending on memory age.

Signaled Recent vs Remote: Aggregated Cue Analysis

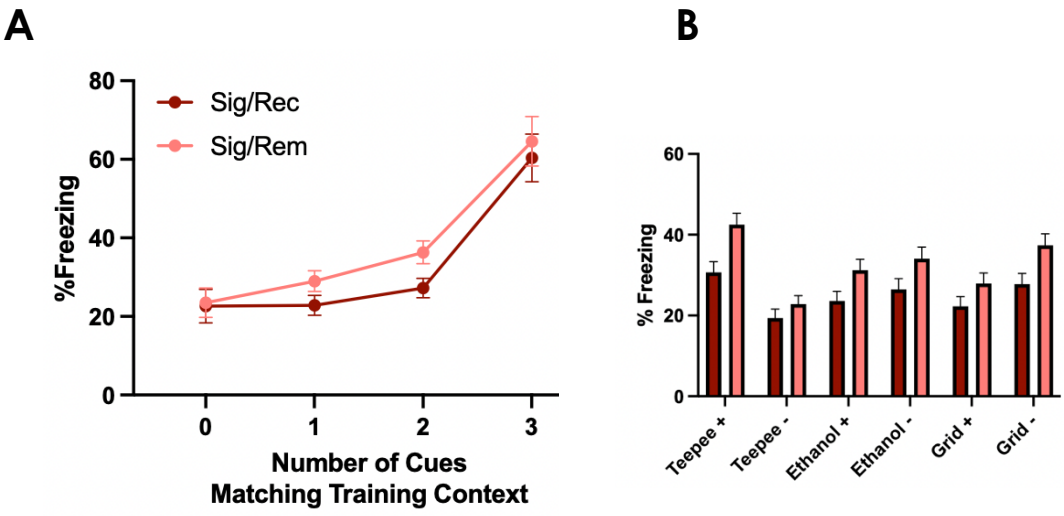

Figure 1.8: A) Aggregated mean context freezing data (from all ten context tests) based on the number of cues matching the original training context. **B)** Relative importance of cues from three sensory modalities: visuospatial, olfactory, and tactile. Aggregated mean freezing data grouped by the presence or absence of the three original training context cues (teepee/visuospatial, ethanol/olfactory, and grid/tactile). Maroon bars represent recent memory and pink bars represent remote memory. Freezing from only the six partial contexts is included. Data are depicted as means +/- SEM. There were no significant differences due to memory age.

Experiment 2: Unsignaled training

Identical statistical analyses were conducted from Experiment 1 (signaled training) to Experiment 2 (unsignaled training). A MANOVA with two within-subjects factors (memory age x context test type) revealed a significant main effect of context test type ($F(9, 207) = 39.61$, $p < 0.001$]. There was no significant main effect of memory age ($F(1,23) = 2.29$, $p = 0.14$; see Fig 1.13]. However, there was a significant interaction between memory age and context test type ($F(9, 207) = 8.76$, $p < 0.001$].

A second MANOVA with two within-subjects factors (memory age x number of cues matching the training context) revealed a significant main effect of the number of cues matching the training context ($F(3, 69) = 177$, $p < 0.01$]. There was no main effect of memory age ($F(1,23) < 0.001$, $p = 1.0$; see Fig 1.14]. Likewise, there was no significant interaction between memory age and number of cues ($F(3,29) < 0.001$, $p = 1.0$].

A third MANOVA with two within-subjects factors (memory age x training cue type) was run using freezing data from only the six partial context tests. Results of this test revealed a significant main effect of training cue type [$F(5,115) = 5.92$, $p < 0.001$]. There was no significant main effect of memory age [$F(1,23) < 0.001$, $p = 0.99$; see Fig 1.14] and no significant interaction between memory age and training cue type [$F(5,115) < 0.001$, $p = 0.99$].

Post-hoc analyses: recent memory following unsignaled (tone-absent) training. After a 24-hour consolidation period following tone-absent conditioning, all mice began a ten-day testing regimen.

Individual Tests: partial contexts versus full training context. Freezing to all partial contexts was significantly lower compared to freezing to both the first and second test of the training context (ts > 4.22, *p*s < 0.01).

Individual Tests: partial contexts versus neutral context. Partial context freezing did not differ significantly from either test of the neutral context (ts < 2.9, *p*s > 0.05), with the exception of the teepee + grid context, which only yielded significantly higher freezing compared to the second presentation of the neutral context [$t(23) = 3.65$, $p = 0.04$].

Individual Tests: Extinction. There was some evidence of extinction, given that freezing to the second presentation of the training context was significantly lower compared to freezing to the first presentation of the training context [$t(23) = 4.35$, $p = 0.02$], with an overall mean difference in freezing percentage of 26.6%.

Individual Tests: Generalization. There was no evidence of generalization to the neutral context, as freezing to the first and second presentations did not differ [$t(23) = 1.31$, $p = 0.99$].

Number of Cues. When memory was assessed within a week of training, mice that underwent tone-absent training froze significantly more to the entire training context (3 cues group) compared to how they froze to contexts with 0,

1, or 2 training cues (*ts* > 10.9, *ps* < 0.001). Freezing scores across the 0, 1, and 2 cue groups were not significantly different (*ts*< 2.24, *ps* > 0.05). Together, these findings provide further evidence that intact mice learn and respond to contextual cues in an all-or-none fashion, regardless of whether the context is in the 'foreground' or 'background' during training.

This data set was also used to assess the goodness of fit of contextual freezing data (after unsignaled training) to custom elemental and configural models (see Table 1.3). This approach determined that the configural model provided a significantly better fit for the observed data for the critical intermediate cue conditions (1 or 2 cues matching the training context). Thus, recent contextual memory following signaled training appears to be represented in a configural manner.

Table 1.3: Goodness of Fit Comparison between custom configural and elemental models for contextual freezing data (recent memory) following unsignaled training. Mean squared errors (MSE), p-value, and observed vs. predicted freezing values for partial contexts with 1 and 2 cues matching the original training contexts are reported. The configural model fit the data significantly better compared to the elemental model ($p < 0.001$).

Unsignaled Recent	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	12.85	12.85	203.42	
Elemental Expected	25.69	38.53	493.97	
Actual Values	12.34	18.73		< 0.001

By cue type. Analysis of freezing (to only the six partial contexts) by cue type revealed a significant effect for only the teepee presence [$t(23) = 3.29$, $p = 0.03$]. However, mice only froze 5.8% more on average to the partial contexts when a teepee was present versus when it was absent.

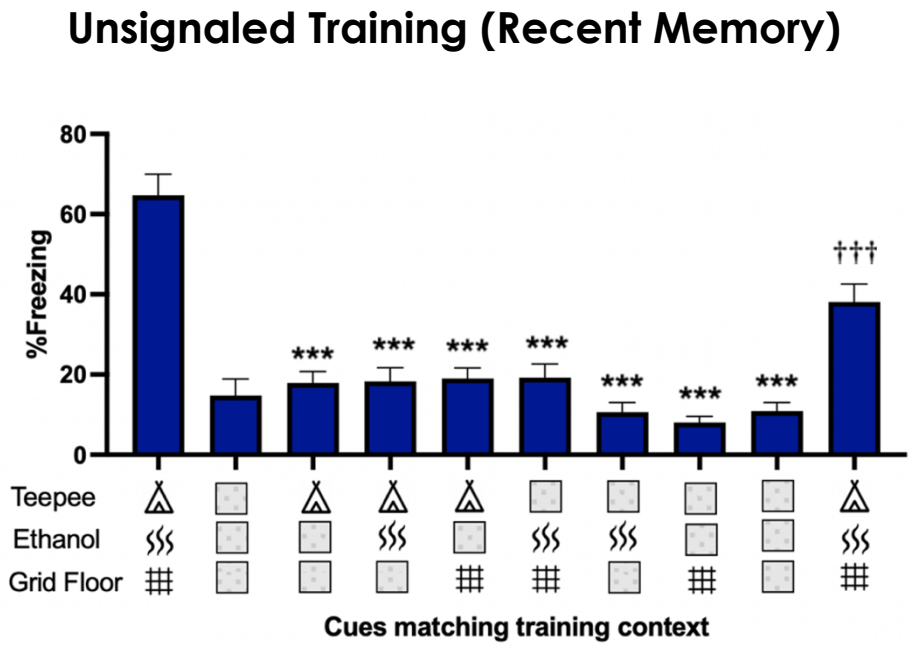

Figure 1.9: Percent freezing (a proxy for context memory) tested 1-10 days after unsignaled (tone-absent) fear conditioning. This 'recent' memory is considered long-term consolidated memory, as synaptic consolidation has occurred, but the memory is not expected to have undergone complete cortical consolidation at this point. One context test was administered each day for ten consecutive days. Each bar represents the mean percent freezing score for each individual context test. Symbols below each bar indicate the cues in each context configuration matching the training context; grey boxes indicate the absence of a given training cue. Asterisks (*) represent significance against the first test of training context memory. Daggers (†) represent significance against the first test of the neutral context. Error bars represent SEM. For each comparison, * or † *Ps* < 0.05, ** or †† *Ps* < 0.01, *** or ††† *Ps* < 0.001.

Unsignaled/Recent: Aggregated Cue Analysis

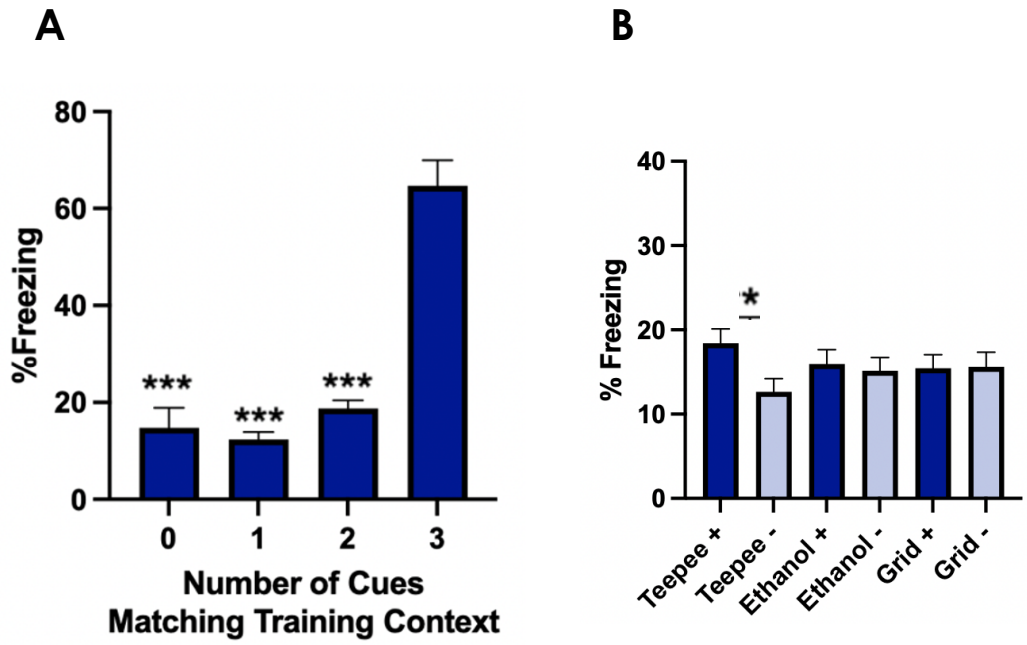

Figure 1.10: A) Aggregated mean context freezing data (from all ten context tests) based on the number of cues matching the original training context. Asterisks represent significant comparisons against the 'three cues' group. The groups with 0, 1, or 2 cues matching the training context did not differ significantly. **B)** Relative importance of cues from three sensory modalities: visuospatial, olfactory, and tactile. Aggregated mean freezing data grouped by the presence or absence of the three original training context cues (teepee/visuospatial, ethanol/olfactory, and grid/tactile). Freezing from only the six partial contexts is included. Data are depicted as means +/- SEM. Asterisks represent significant comparisons between the presence and absence groups for each cue type. For each comparison, * *Ps* < 0.05, ** *Ps* < 0.01, ** *Ps* < 0.001.

Post-hoc analyses: remote memory following unsignaled (tone-absent) training. Mice were retested 50 days after the initial testing to determine if generalization or any change in responsivity to the various cues was seen.

Individual Tests: partial contexts versus training context. Mice only froze significantly less for two of the partial contexts (grid only and ethanol only) compared to the first presentation of the training context at the remote timepoint (*ts* > 4.39, *ps* <0.01). This pattern held when comparing the partial contexts against the second presentation of the training context at the remote timepoint (*ts* > 4.63, *ps* < 0.01). These results suggest that mice showed some difficulty distinguishing the training context from contexts that either included the teepee cue or contained two out of the three cues present in the original training environment.

Individual Tests: comparison against first neutral context test. For the most part, mice did not freeze differently to the first presentation of the neutral context compared to the partial contexts (*ts* > 2.78, *ps* > 0.05). However, their freezing was significantly higher to the teepee only and teepee + ethanol contexts compared to the first presentation of the neutral context (*ts* > 4.24, *ps* < 0.01). On the other hand, freezing to second presentation of the neutral context did not differ compared to freezing for any of the partial contexts (*ts* < 2.34, *ps* > 0.05).

Individual Tests: Extinction. There was no significant decrease in freezing from the first to the second presentation of the training context [$t(23) = 0.54$, $p = 0.99$].

Individual Tests: Generalization. There was a significant increase in freezing from the first to the second presentation of the neutral context [$t(23) = 3.72$, $p = 0.03$].

Number of Cues. At the remote timepoint, mice froze significantly more for the full training context (with all three cues present) compared to all other contexts (with 0, 1, or 2 cues present; $ts > 4.23$, $ps < 0.01$).

While the MANOVA results suggest a configural representation still holds at the remote timepoint, the goodness of fit analysis (see Table 1.4) suggests that the data do not fit significantly better to either the elemental or the configural model. This demonstrates a trend away from the strict configural representation at the remote timepoint after unsignaled training, suggesting that by this time memory could be beginning to change toward elemental responding.

Table 1.4: Goodness of Fit Comparison between custom configural and elemental models for contextual freezing data (remote memory) following unsignaled training. Mean squared errors (MSE), p-value, and observed vs. predicted freezing values for partial contexts with 1 and 2 cues matching the original training contexts are reported. Neither the configural nor the elemental models yielded a significantly better fit to the data.

Unsignaled Remote	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	18.97	18.97	359.54	
Elemental Expected	25.05	31.13	381.35	
Actual Values	21.22	24.65		0.51

By cue type. For the partial context tests, mice froze significantly more overall to contexts with a teepee compared to those without [$t(23) = 5.97$, $p < 0.001$]. On average, mice froze 11% more for the partial contexts when the teepee was present.

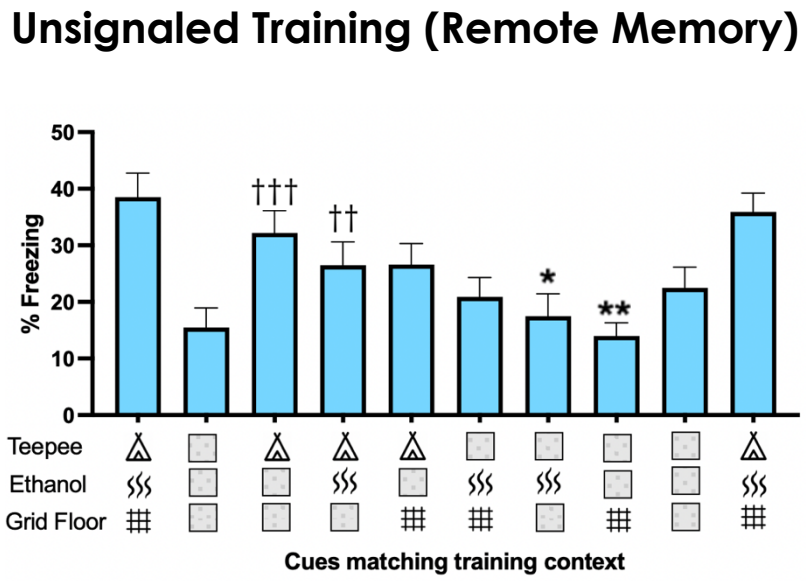

Figure 1.11: Percent freezing (a proxy for context memory) tested 50 days after unsignaled (tone-absent) fear conditioning. This 'remote' memory is considered long-term consolidated memory, well after cortical consolidation has occurred. One context test was administered each day for ten consecutive days. Each bar represents the mean percent freezing score for each individual context test. Symbols below each bar indicate the cues in each context configuration matching the training context; grey boxes indicate the absence of a given training cue. Asterisks (*) represent significant comparisons against the first test of training context memory. Daggers (†) represent significant comparisons against the first test of the neutral context. Error bars represent SEM. For each comparison, * or † *Ps* < 0.05, ** or †† *Ps* < 0.01, *** or ††† *Ps* < 0.001.

Unsignaled/Remote: Aggregated Cue Analysis

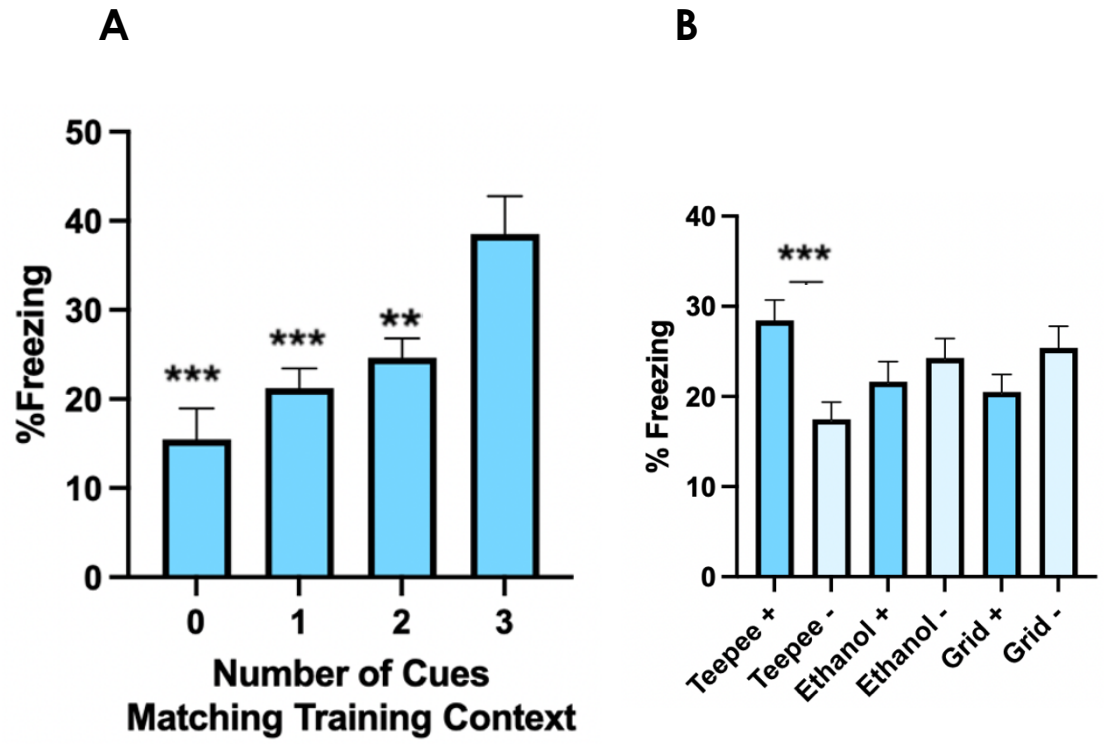

Figure 1.12: A) Aggregated mean context freezing data (from all ten context tests) based on the number of cues matching the original training context. Asterisks represent significant comparisons against the 'three cues' group. **B)** Relative importance of cues from three sensory modalities: visuospatial, olfactory, and tactile. Aggregated mean freezing data grouped by the presence or absence of the three original training context cues (teepee/visuospatial, ethanol/olfactory, and grid/tactile). Freezing from only the six partial contexts is included. Data are depicted as means +/- SEM. Asterisks represent significant comparisons between the presence and absence groups for each cue type. For each comparison, * *Ps* < 0.05, ** *Ps* < 0.01, *** *Ps* < 0.001.

Unsignaled Training (Recent vs Remote Memory)

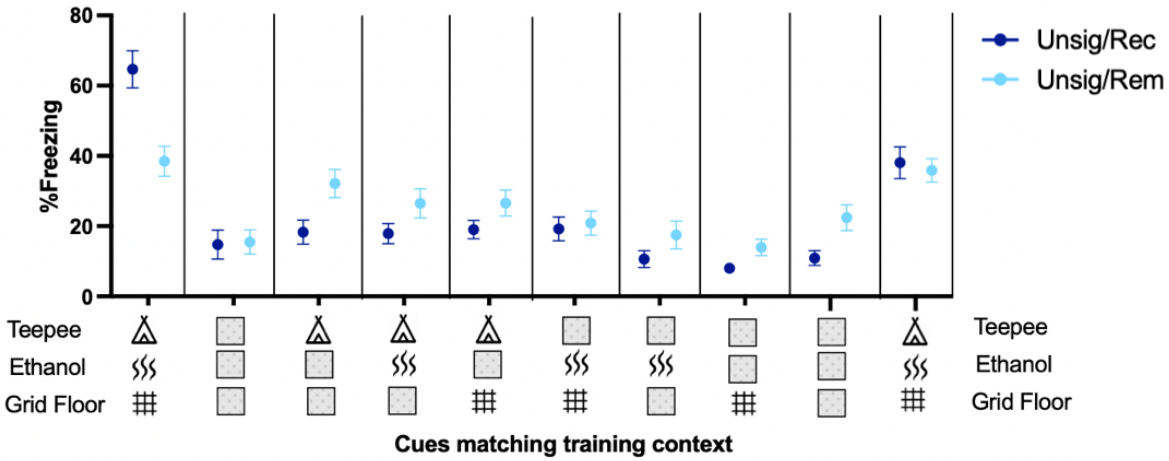

Figure 1.13: Comparison of recent and remote memory after unsignaled training. Dark blue symbols represent recent memory (Unsig/Rec) and light blue symbols represent remote memory (Unsig/Rem). Symbols below each set of recent and remote points indicate the cues in each context configuration matching the training context; grey boxes indicate the absence of a given training cue. Memory did not differ significantly as a function of age in the unsignaled trained group of mice, with the exception of a significant decrease in freezing between recent and remote time points for the first presentation of the training context ($p < 0.01$).

Unsignaled Recent vs Remote: Aggregated Cue Analyses

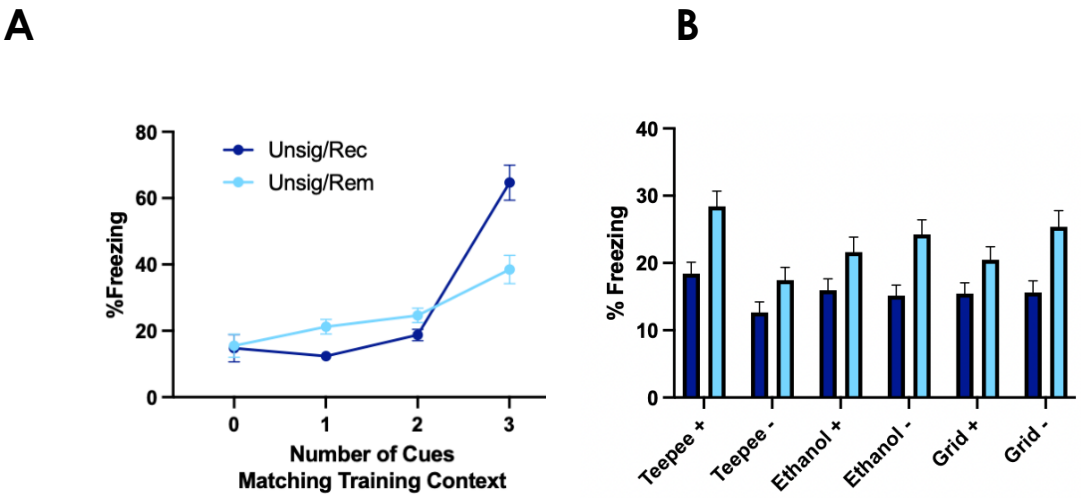

Figure 1.14: A) Aggregated mean context freezing data (from all ten context tests) based on the number of cues matching the original training context, comparing recent and remote time points following unsignaled training. **B)** Relative importance of cues from three sensory modalities: visuospatial, olfactory, and tactile. Aggregated mean freezing data grouped by the presence or absence of the three original training context cues (teepee/visuospatial, ethanol/olfactory, and grid/tactile). Dark blue bars represent recent memory, and light blue bars represent remote memory following unsignaled training. Freezing from only the six partial contexts is included. Data are depicted as means +/- SEM.

Discussion

In this study, we found remarkable support for the view that contextual fear conditioning is supported by configural/spatial learning rather than elemental learning in intact animals. Mice exhibited strong conditioning to the context, but showed little evidence of fear to any of its components presented individually. This was true even after signaled and unsignaled training or after fifty days in signaled mice. However, there was some evidence that contextual memory become somewhat more elemental after two months after unsignaled training.

Wiltgen & Silva (2007) found that generalization began to occur in hybrid [C57BL6/NTac (Taconic) x 129SvE (Jackson)] mice after a period of 36 d. They suggested that context memories are specific early after training when they require the hippocampus and become more general as they are permanently stored in the cortex (Frankland et al., 2001; Frankland and Bontempi, 2005). However, the mice in that study only received 1 tone-shock pairing during testing, and were a different 129B6 hybrid strain. Evidence that several varieties of B6 x 129 hybrid mice perform very similarly on this task (Matynia et al., 2008) suggests that this difference with our findings could be due to their use of a single conditioning trial, but this requires further exploration.

It is possible that the more extensive training for the mice in our study (3 signaled or 2 unsignaled shocks) contributed to their remarkably stable freezing patterns over time, although it is also possible that this difference is due to the

different hybrid stain. Nonetheless, mice appear to generally favor a configural strategy to initially acquire contextual memory and learning remained specific to the training context in Wiltgen & Silva (2007) for about two weeks. However, in our study, contexutal memory is still much more specific as they were comparing very dissimilar contexts.

More extensive training may allow an animal to form a contextual representation that is stable over time, well after memories are consolidated. This representation appears to be somewhat more stable when the training is signaled. Although individual cues are relatively unimportant on their own, visuo-spatial cues such as the teepee may be more salient compared to cues of other sensory modalities. It is possible that the relative importance of a visuo-spatial cue may increase when a brain damaged animal must rely on an elemental strategy to form a contextual representation.

Traditionally, studies of contextual fear memory have followed the procedure of training with tone-shock pairings with a relatively brief time period between training and testing. This conventional procedure was employed in the Sig/Rec trials. For these signaled trials, it appears the mice used a configural strategy to acquire a representation of the context. They did not display a strong response to the individual contextual cues and only demonstrated a robust freezing response when exposed to all three original training cues. If any of the original training cues were missing from the context, they exhibited little or no freezing response. This is consistent with views supporting the configural strategy

as dominant (Young, Bohenek, & Fanselow, 1994; Frankland et al., 1998; Anagnostaras, Gale, & Fanselow, 2001; Rudy, Huff, & Matus-Amat, 2004; Rudy & Wright-Hardesty, 2005), although this experiment is the first direct test of those views. This heightened fear response to the original training context remained stable (without extinction), even after much repeated testing of similar contexts.

The stability after repeated testing is a bit unexpected, as Biedenkapp & Rudy (2007) found that rats froze similarly when tested on a subset of training condition cues as they did for the original training condition, although that study used rats. Aside from stability over the course of various testing, the mice exhibited an impressively stable response after a substantial time delay (two months) from the original training. This indicates that the configural representation held up after the memories were consolidated and responsibility moved from the hippocampus to cortical areas. Only slight degradation took place by the time the mice were tested on the second remote test of the training context.

Mice also appeared to pay little attention to the individual cues after Unsignaled training. In fact, these mice exhibited an even stronger configural strategy; they displayed a more pronounced difference in freezing between the training context and all other contexts compared to the signal-trained mice. This finding questions the interpretation in Phillips and LeDoux (1994) that an elemental strategy may be utilized when the context is brought into the foreground, although two months later contextual memory appears somewhat

more elemental in this group. Nonetheless, it appears that intact mice will utilize a configural strategy even when the context is brought into the attentional foreground and training is robust, but it may degrade more rapidly than after signaled training.

It does appear that the visuo-spatial contextual cue became more important over time in the unsignaled group. The mice also generally froze more to this cue after unsignaled training compared to signaled training. Visuo-spatial cues may be especially important, as there is evidence to suggest that the hippocampus is dedicated to producing spatial maps (O'Keefe & Dostrovsky, 1971). If the hippocampus is indeed constructing a spatial map to form a memory of the context, it makes sense that a visuo-spatial cue would be more salient than a tactile or olfactory cue. Although the differences were not significant, the signaled mice also exhibited higher levels of freezing on average to the partial contexts with the visuo-spatial training cue compared to the partial contexts that lacked this cue.

Thus, mice appear to generally favor a configural strategy to form a contextual memory and the individual contextual cues are of relatively little importance on their own. Extensive training allows an animal to form a contextual representation that is stable over time, well after memories are consolidated. This representation appears to be somewhat more stable when the training is signaled. Although individual cues are relatively unimportant on their own, visuo-spatial cues may be more salient compared to cues of other

sensory modalities. It is possible that the relative importance of a visuo-spatial cue may increase when a brain damaged animal must rely on an elemental strategy to form a contextual representation. Overall, data from this experiment provide impressive support for the configural view of contextual fear conditioning, at least in our strain of hybrid mice. Further research will be required to examine if this pattern holds up under different training parameters (especially one trial learning) or in other animals (e.g., rats or other lines of mice).

Acknowledgments

Chapter 1 includes material that is currently being prepared for submission for publication: Van Alstyne, K. & Anagnostaras, S. (in prep). The dissertation author was the primary researcher and author of this material.

CHAPTER TWO

Building contexts on shaky foundations: the effects of drugs with amnesic properties on contextual memory representations

In this chapter, I sought to explore whether degradation of contextual memory acquisition by various amnesic drugs could tip the balance of learning between configural and elemental representations. We chose two canonical amnesic drugs, CPP [(±)-3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid], an NMDA receptor antagonist, and scopolamine, a cholinergic muscarinic antagonist, which are both well characterized in this paradigm, and argued to disrupt hippocampal function (Anagnostaras et al., 1999; Carmack et al., 2013; Anagnostaras et al., 2003; Laha et al., 2022), and one novel NMDA receptor antagonist, esketamine. For CPP and scopolamine, we examined both non-amnesic and amnesic doses, while esketamine was characterized more broadly. All of these drugs may also produce perceptual disturbance, which we hypothesized could be more detrimental to contextual learning than to discrete cue learning (Jelen et al. 2021).

Scopolamine is a competitive cholinergic muscarinic receptor antagonist and produces amnesia that is selective for contextual as opposed to discrete or cued (tone) learning (Anagnostaras et al., 1999). Likewise, intrahippocampal infusion of scopolamine administered pre-training produces a selective amnesia for contextual fear (Gale et al., 2001). Furthermore, Rudy (1996) and Pavesi et al. (2012) have both reported that scopolamine impaired discrimination among

similar CSs. Together, these results suggest that scopolamine could shift mice from a configural to an elemental strategy as dose increases, because of a disruption of hippocampal functioning and/or perceptual difficulty.

CPP is a potent competitive NMDA receptor antagonist (at the glutamate and AP5 site; Davies et al., 1986) which produces a nonselective amnesia for contextual and tone fear (Carmack et al. 2013; Laha et al., 2022); because NMDA receptor antagonism disrupts long-term potentiation, context learning, and cued fear conditioning, systemic administration of CPP was expected to impair conditioning very generally (Quinn et al., 2005; Laha et al., 2022).

Finally, esketamine is a constituent isomer of ketamine, a non-competive antagonist of NMDA receptors at the allosteric phencyclidine site (Jelen et al., 2020). In contrast to CPP and scopolamine, the effects of esketamine on fear conditioning have not been previously investigated, although racemic ketamine has been demonstrated to be a potent amnesic in fear conditioning (Pietersen et al., 2006). Esketamine differs from racemic ketamine in important ways. For example, it is a more potent NMDA receptor antagonist and has reduced binding at the hallucinogenic and antinociceptive sigma receptor (Jelen et al., 2020). Because esketamine is a potent NMDA receptor antagonist, it might be expected to generally impair conditioning, similarly to what is expected of CPP. Alternatively because dissociation and perceptual disturbance has been reported occasionally in humans (Janssen, 2023), it is

possible that it may cause a shift toward an elemental strategy with increasing dose, as perception becomes more difficult.

Materials and Methods

Animals

A total of 223 (114 male and 109 female) hybrid C57BL/6Jx129S1/SvImJ (129B6; Jackson Laboratory, West Sacramento, CA, USA) mice were used. This mouse strain was selected based on recommendations made at the Banbury Conference on Genetic Background in Mice, which was held to facilitate the comparison of results between experiments and among laboratories. This strain is often selected because of its strong performance on behavioral memory measures (Silva et al. 1997). Mice were weaned at 3 weeks of age and group-housed (2–5 mice per same-sex cage) with unrestricted access to food and water. The animal colony was maintained on a 14:10-h light/dark schedule, and all experimental procedures occurred during the light phase. Mice were at least 10 weeks old and handled for 3 days (1 min/day) prior to fear conditioning and testing. All animal care and experimental procedures were approved by the UCSD IACUC and compliant with the NRC Guide for the Care and Use of Laboratory Animals.

Drugs

Experiment 1. Scopolamine HBr (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9 % saline and given intraperitoneally (i.p.) in a volume of 10 mL/kg. A range of scopolamine doses was selected: 0.25, 0.5, 1, and 5 mg/kg

ranging from sub-amnesic to moderately amnesic (salt weight; Anagnostaras et al., 1999; Anagnostaras et al., 2003).

Experiment 2. (RS)-CPP ([±3-(2-carboxy-piperazine-4-yl)-propyl-1-phoshonic acid; Tocris Bioscience, Bristol, UK] was dissolved in 0.9 % saline and given intraperitoneally (i.p.) in a volume of 10 mL/kg. A range of (RS)-CPP doses was selected ranging from sub-amnesic to moderately amnesic: 1, 5, and 10 mg/kg (salt weight; Carmack et al., 2013).

Experiment 3. (S)-Ketamine HCl (also known as esketamine; Cayman Chemical, Ann Arbor, MI, USA) was dissolved in 0.9 % saline and given intraperitoneally (i.p.) in a volume of 10 mL/kg. A range of (S)-Ketamine doses was selected: 1, 5, and 10 mg/kg (salt weight).

Fear Conditioning

The well-validated VideoFreeze software (Med-Associates Inc., Georgia, VT, USA) captured and scored each individual mouse's freezing behavior from an infrared camera (Anagnostaras et al. 2000, 2010). This program controlled the tones and shocks to the chambers. Up to seven mice were trained and tested simultaneously in individual fear conditioning chambers (32 x 25 x 25 cm), which consisted of stainless-steel sidewalls, white acrylic back walls, and clear polycarbonate front and top walls. Each chamber was transformed across three sensory dimensions to create distinct contexts (cues described below).

Context Cues

For all mice, in both *Experiment 1* and *Experiment 2,* all contexts consisted of three cues of three different sensory modalities (olfactory, visuo-spatial, and tactile). The full training context consisted of a strong 50% ethanol odor (olfactory), illuminated with a moderate (80 lux) white house light and triangular teepee (visuo-spatial), and a stainless-steel grid floor (tactile). The neutral context consisted of a weak (7% and rinsed with water) isopropanol odor (olfactory), no visible light or teepee (visuo-spatial), and a white acrylic floor (tactile). Examples of the training and neutral contexts are depicted in Figure 1.2. Prior to placing a mouse into the chamber, all surfaces within the space were cleaned with an odor matching the intended olfactory cue: either 50% ethanol or 7% isopropyl alcohol rinsed with water to minimize any residual odor, and wiped dry. Extra care was taken to ensure the strong ethanol odor did not linger in a chamber presentation intended to include the weak olfactory cue.

Experimental Procedures

Fear Conditioning Training

Saline controls. As all three experiments (scopolamine, CPP, and esketamine) were run concurrently, the saline controls were pooled into one group and used for comparison in each experiment. There were a total of 50 control mice.

Experiment 1. A total of 56 mice were randomly assigned to groups by dose of Scopolamine-HBr administered: 0.25 (n = 18), 1 (n = 18), and 5 (n = 20)

mg/kg. Groups were counterbalanced by sex and conditioning chamber. Mice were given an i.p. injection of scopolamine or saline 20 min before a 5-min fear conditioning session. A delay of 20 min was selected due to reported peak amnesic effect timing (Busquet et al., 2012).

Experiment 2. A total of 54 mice were randomly assigned to groups by dose of CPP administered: 1 (n = 18), 5 (n = 18), and 10 (n = 18) mg/kg. Groups were counterbalanced by sex and conditioning chamber. Mice were given an injection of CPP or saline 20 min before a 5-min fear conditioning session. A delay of 20 min was selected due to the reported peak amnesic effect timing (as in Carmack et al., 2013).

Experiment 3. A total of 48 mice were randomly assigned to groups by dose of esketamine administered: 1 (n = 15), 5 (n = 15), and 10 (n = 18) mg/kg. Groups were counterbalanced by sex and conditioning chamber. Mice were given an injection of esketamine or saline 15 min before a 5-min fear conditioning session. A delay of 15 min was selected due to its coincidence with peak drug effect (based on locomotor activity pilot data from our lab; data not shown).

Mice were placed inside the chamber with all the training cues (50% ethanol odor, house light and black teepee insert, and grid floor) present. The mice were recorded for 2 minutes prior to tone-shock pairings for baseline freezing and locomotor activity measures. The mice were given this pre-shock time period to allow them to adequately explore their environment, preventing

the immediate shock deficit (Fanselow, 1986, 1990; Wiltgen et al., 2006; McHugh & Tonegawa, 2007; Frankland et al., 2004). Following the baseline period, the mice experienced three tone-shock pairings. Each pairing consisted of a single 30s tone (2.8kHz, 85dB) that co-terminated with a 2s foot shock (0.75 mA, RMS AC constant current) that was transmitted through the grid floor. The interval between pairings and after the final pairing was 30s. Thus, the mice were in the chamber for a total of 5 min during training. VideoFreeze software scored freezing and locomotor activity throughout the entire session.

Context testing

Beginning the day after training, all mice (across all three experiments) were tested (off-drug) on one context per day for ten consecutive days. On day one, all mice were tested on the neutral context, which is described above. On day two, all mice were tested on the original training context, also described above. The first two days consisted of baseline maximum responding to the training context and baseline minimum responding to the neutral context. On days 3-8, mice were tested on one of six partial contexts, where one or two cues matched the original training context, and the remaining cue(s) were from the neutral context. Partial contexts were counterbalanced over these six days. In order to evaluate the effects of repeated testing (for example, to determine if extinction occurred), two additional anchoring tests were completed on days 9 and 10. On the ninth day, all mice were retested on the neutral context, and on the tenth day all mice were retested on the full original training context. For

each test, mice remained in the chamber for 5 minutes without experiencing any tones or shocks. Freezing was scored for 5 minutes to measure the cumulative effect of individual contextual cues on fear memory.

Statistical Analyses

Univariate ANOVAs were conducted to measure differences in baseline locomotor activity and shock reactivity (measured in arbitrary units, Au; see Anagnostaras et al., 2010 for details) across drug condition. The mean differences in context freezing data for this set of experiments were analyzed using multivariate analyses of variance (MANOVAs) with one or two within-subjects factors (name) and one between subjects factor (drug condition). In each analysis, percent freezing served as the dependent measure. Data from male and female mice were collapsed, as no statistically significant differences were found based on sex ($ps > 0.05$). Post hoc comparisons were performed following significant group differences using Tukey's Honest Significant Difference (HSD) test. All data are presented as means ± standard error the mean (SEM). ANOVAs/MANOVAs and post-hoc analyses were conducted in Jamovi (version 2.5), an open-source statistical analysis software. Graphical representations of the data were created using Graphpad Prism 9 (GraphPad Software, LLC).

To compare the fit of each condition's (i.e., controls, individual drug dosages) data to custom configural and elemental models of contextual fear conditioning, a permutation test focusing on the predictive accuracy for

intermediate cue conditions (1 and 2 matching contextual cues) was employed. These analyses were performed using R (version 4.2; R Core Team, 2024) with the dplyr package. See Chapter 1 for full details on this method of analysis.

Results

Saline group characterization across individual context tests

A repeated-measures ANOVA was conducted to characterize the differences in freezing across individual context test for the saline controls. There was a significant main effect of test type [$F(9, 441) = 71.5$, $p < 0.001$]. All partial contexts were significantly lower on freezing compared to both tests of the full training context ($ts > 5.32$, $ps < 0.001$), mirroring the results of the data from Chapter 1.

When partial contexts were compared against both tests of the neutral context, the three partial contexts containing the teepee cue yielded significantly higher freezing ($ts > 4.27$, $ps < 0.01$). However, the mice only froze about 14% more to the partial contexts with a teepee, which is a relatively small contribution considering the mice freeze on average 26.5% to the novel neutral context alone.

There was a significant difference between the first and second tests of the training context ($t(49) = 5.76$, $p < 0.001$), suggesting that some extinction occurred. However, despite this effect, it is important to note that freezing to the

second test of the training context was still significantly higher than freezing to all partial contexts (*ts* > 5.32, *ps* < 0.001).

There was no significant difference on freezing from the first to the second test of the neutral context [$t(49) = 0.76$, $p = 0.99$], suggesting no generalization occurred after repeated exposure to the partial contexts containing some neutral cues, which also parallels the pattern seen in the Chapter 1 experiments.

Elemental versus configural representation. Using a bootstrapping method to determine whether a custom configural or elemental model provided a significantly better fit to the saline data, results demonstrated that the configural model provided a significantly better fit (See Table 2.1). Similarly to the intact mice used in Chapter 1 experiments, saline controls for the Chapter 2 experiments appear to represent context memory in a configural manner.

Table 2.1: Goodness of Fit Comparison between custom configural and elemental models for contextual freezing data from saline controls. Mean squared errors (MSE), p-value, and observed vs. predicted freezing values for partial contexts with 1 and 2 cues matching the original training contexts are reported. The configural model provided a significantly better fit to the data over the elemental model.

Saline controls	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	26.57	26.57	629.24	
Elemental Expected	39.65	52.73	760.63	
Actual Values	30.61	37.86		< 0.001

By cue type. For partial context tests, saline controls froze significantly more when the teepee was present versus absent [$t(49) = 7.69$, $p < 0.001$]. They

did not respond to the partial contexts differently depending on the presence or absence of either the ethanol or grid cues (*ts* < 0.95, *ps* > 0.05).

Baseline locomotion

To determine if any drug dosages affected baseline locomotor activity differently than saline controls, three separate one-way ANOVAs were conducted, one for each drug (scopolamine, CPP, and esketamine). The dependent variable was the average locomotor activity during the 2-minute baseline period on training day. These tests were performed to rule out potential confounds in freezing data caused by high baseline locomotion.

The ANOVAs revealed no significant differences across drug dosages compared to saline controls (all *Fs* < 3.1, all *ps* > 0.05), except the 10 mg/kg esketamine group had significantly lower ($t = 3.5$, $p < 0.01$) baseline activity compared to the saline control group.

Shock reactivity

To determine if any drug dosages affected shock reactivity (average activity during shock transmission) compared to saline controls, three separate one-way ANOVAs were conducted for each drug. These tests were performed to rule out potential analgesic effects from the drugs that could confound the freezing data. The ANOVAs revealed no significant differences across drug dosages compared to saline controls (all *Fs* < 4.2, all *ps* > 0.05), except the 0.25 mg/kg scopolamine group had significantly higher ($t = 3.17$, $p = 0.02$) shock reactivity compared to the saline control group.

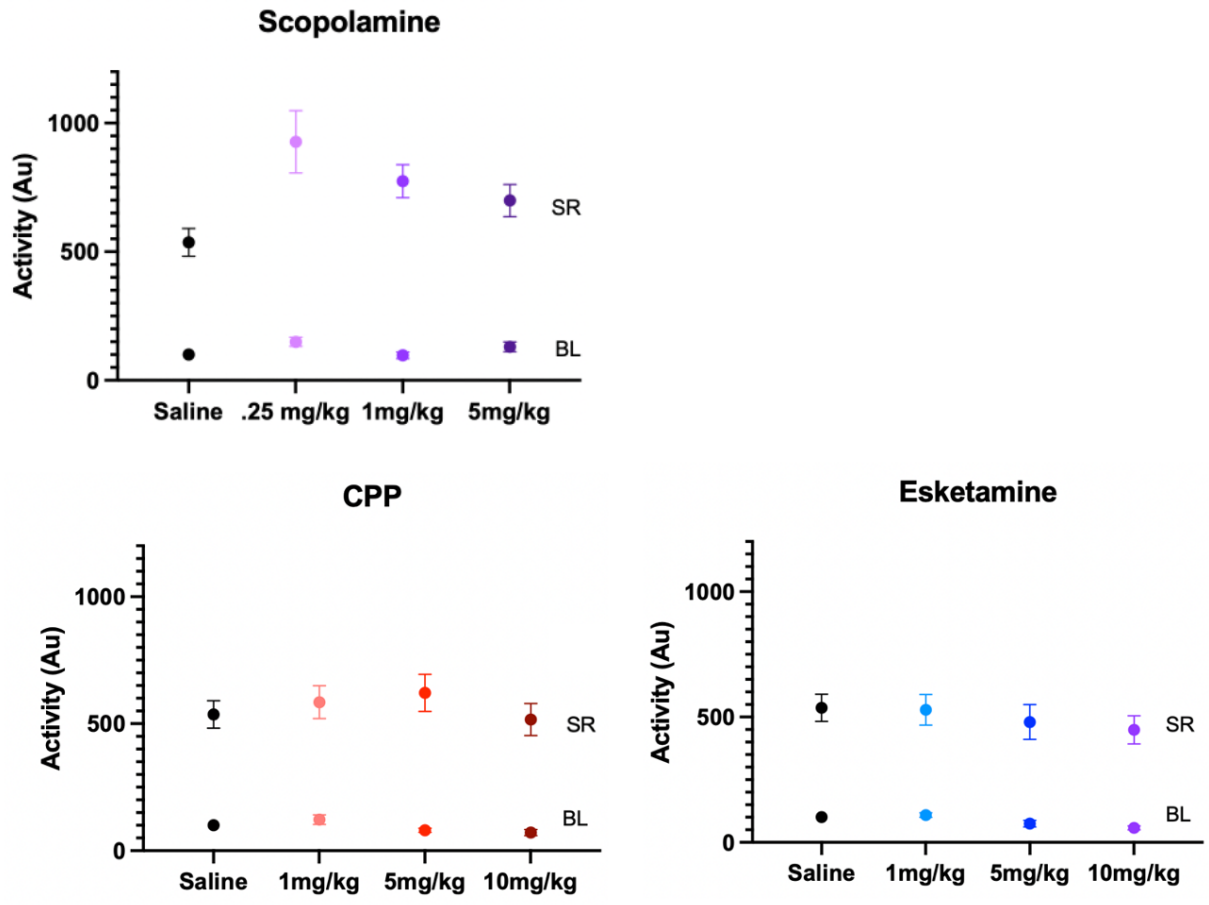

Figure 2.1: Baseline locomotor activity during the training day and average shock reactivity across all three training shocks were analyzed to identify potential confounds in the drug-induced amnesic effect. This analysis was used to rule out alternative explanations for reduced freezing scores observed from context tests. Specifically, the aim was to determine whether lower context freezing scores could be genuinely ascribed to an amnesic effect of the drugs, rather than resulting from either decreased shock reactivity or increased baseline locomotor activity. Neither effect was shown across drug dose or drug type, suggesting that any reductions in context freezing are amnesic effects.

Scopolamine

A MANOVA with one within-subjects factor (individual context test) and one between-subjects factor (drug condition) revealed a significant main effect of context test type [$F(9,918)= 82.02$, $p < 0.001$]. There was also a significant main effect of drug condition [$F(3,102) = 3.16$, $p = 0.03$] and a significant interaction between context test and drug condition[$F(27,918)= 1.99$, $p < 0.01$].

Comparison against controls across all partial contexts. Post hoc comparisons were conducted for each partial context test comparing drug groups against the saline controls. However, no significant differences were found ($ts < 2.8$, $ps > 0.05$).

Number of Cues. A traditional analysis (MANOVA) was conducted first, revealing a significant main effect of number of cues matching the training context (within-subjects factor): [$F(3,306) = 285.01$, $p < 0.001$], a significant main effect of drug condition (between-subjects): [$F(3,102) = 3.61$, $p = 0.02$], and a significant interaction: [$F(9, 306) = 2.7$, $p = 0.01$]. Post hoc analyses are featured in table 2.5. However, a more sensitive bootstrapping approach was employed to determine whether the data (aggregated based on the number of cues matching the training context) fit with a more elemental or more configural representation of the context by drug dose. The goodness of fit of custom elemental and configural models for the scopolamine data revealed a dose-dependent shift to an elemental strategy, at doses well below those were amnesic effects are apparent (see Table 2.2).

Table 2.2: Goodness of Fit Comparison between custom configural and elemental models for contextual freezing data across doses of **scopolamine** (including saline controls for reference). Mean squared errors (MSE), p-value, and observed vs. predicted freezing values for partial contexts with 1 and 2 cues matching the original training contexts are reported. Results of these analyses indicate that mice exhibit a dose-dependent shift from a configural to a more elemental representation of context when trained on scopolamine.

Saline controls	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	26.57	26.57	629.24	
Elemental Expected	39.65	52.73	760.63	
Actual Values	30.61	37.86		< 0.001
0.25 mg/kg	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	23.42	23.42	594.3	
Elemental Expected	35.47	47.53	566.98	
Actual Values	27.58	36.15		0.49
1 mg/kg	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	20.82	20.82	442.92	
Elemental Expected	31.04	41.26	426.82	
Actual Values	22.38	33.88		< 0.001
5 mg/kg	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	11.99	11.99	559.69	
Elemental Expected	22.92	33.85	428.01	
Actual Values	19.09	30.19		< 0.001

Comparison of full training context memory by dose. A comparison of freezing responses across drug conditions for the '3 cues' group revealed a significant reduction in freezing for only the 5 mg/kg group compared to saline controls [$t(102) = 4.04$, $p = 0.01$].

Comparison of neutral context freezing by dose. A comparison of freezing responses across drug conditions for the '0 cues' group revealed no significant differences ($ts < 3.45$, $ps > 0.05$). This indicates that, on average, mice did not freeze differently to the neutral context across scopolamine dosages (including saline controls).

By cue type. A MANOVA with one within-subjects (cue type presence or absence) factor and one between-subjects (drug condition) factor revealed a significant main effect of cue type [$F(5,510) = 14.22$, $p < 0.001$]. There was no significant main effect of drug condition [$F(3,102) = 1.64$, $p = 0.18$], nor was there a significant interaction between cue type and drug condition [$F(15,510) = 1.58$, $p = 0.08$]. Despite the lack of a significant interaction, post hoc analyses were run to investigate the relationship between cue type across drug dose, as prior results (Chapter 2) suggest an a priori prediction that salience of the individual cue types could be an important factor, particularly for the teepee stimulous.

This analysis revealed that the main effect of cue type was largely being driven by controls, as there was a significant difference in freezing when the teepee was present versus when it was absent for the saline group [$t(102) = 7.3$, $p < 0.001$] and for the lowest dosage scopolamine group [$t(102) = 3.93$, $p = 0.03$],

but there was no difference in freezing depending on teepee presence for the 1mg/kg and 5 mg/kg scopolamine groups (*ts* < 2.3, *ps* > 0.05). Furthermore, the saline group (which had a larger effect of teepee presence compared to the 0.25 mg/kg scopolamine group) only froze 11.46% more when the teepee was present versus when it was absent from a partial context, which is not a very large difference, given that the average freezing to the completely neutral context for the saline group is over 20%.

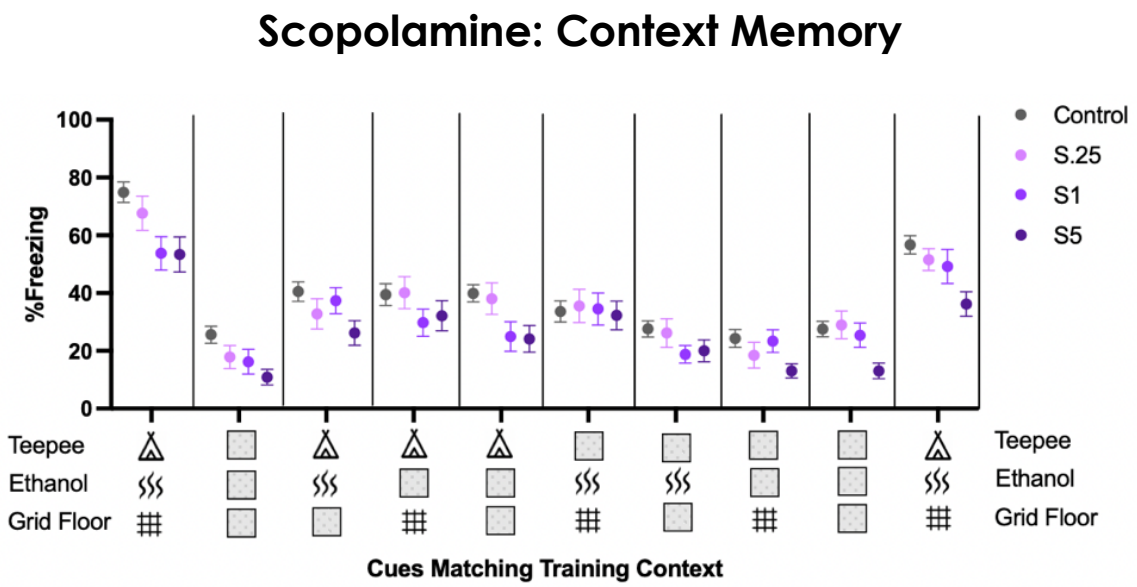

Figure 2.2: Comparison of the effects of a range of scopolamine doses given during training on long-term individual context tests. Symbols below each set of recent and remote points indicate the cues in each context configuration matching the training context; grey boxes indicate the absence of a given training cue. Error bars represent SEM.

Scopolamine: Aggregated Cue Analyses

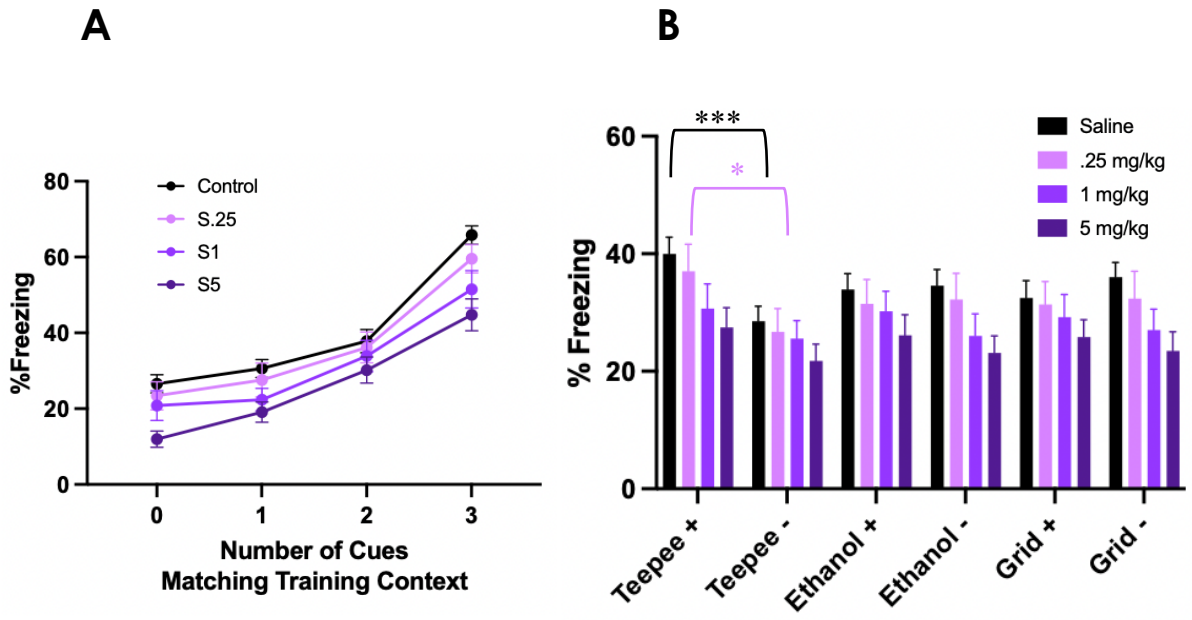

Figure 2.3: A) Aggregated mean context freezing data (from all ten context tests) based on the number of cues matching the original training context. See Table 2.5 for details on MANOVA post hoc analyses. **B)** Relative importance of cues from three sensory modalities: visuospatial, olfactory, and tactile. Aggregated mean freezing data grouped by the presence or absence of the three original training context cues (teepee/visuospatial, ethanol/olfactory, and grid/tactile). Freezing from only the six partial contexts is included. Data are depicted as means +/- SEM. Asterisks represent significant comparisons between the presence and absence groups for each cue type. For each comparison, * *Ps* < 0.05, ** *Ps* < 0.01, ** *Ps* < 0.001.

CPP

A MANOVA with one within-subjects factor (individual context test) and one between-subjects factor (drug condition) revealed a significant main effect of context test type [$F(9,900) = 75.61$, $p < 0.001$]. There was also a significant main effect of drug condition [$F(3,100) = 5.26$, $p < 0.01$] and a significant interaction between context test and drug condition[$F(9,300) = 6.03$, $p < 0.001$].

Comparison against controls across all partial contexts. Post hoc comparisons were conducted for each partial context test comparing drug groups against the saline controls. However, no significant differences were found ($ts < 2.53$, $ps > 0.05$).

Number of Cues. A traditional analysis (MANOVA) was conducted first, revealing a significant main effect of number of cues matching the training context (within-subjects factor): [$F(3,300) = 227.86$, $p < 0.001$], a significant main effect of drug condition (between-subjects): [$F(3,100) = 5.99$, $p < 0.001$], and a significant interaction: [$F(9,300) = 6.03$, $p < 0.001$]. Post hoc analyses are featured in table 2.5. However, a more sensitive bootstrapping approach was employed to determine whether the data (aggregated based on the number of cues matching the training context) fit with a more elemental or more configural representation of the context by drug dose. The goodness of fit of custom elemental and configural models for the CPP data revealed that a configural representation prevailed but conditioned responding became more elemental at the 10 mg/kg dose, where the amnesic effect is profound (see Table 2.3).

Table 2.3: Goodness of Fit Comparison between custom configural and elemental models for contextual freezing data across doses of **CPP** (including saline controls for reference). Mean squared errors (MSE), p-value, and observed vs. predicted freezing values for partial contexts with 1 and 2 cues matching the original training contexts are reported. Results of these analyses indicate that mice trained on CPP represent the context configurally on long-term memory tests until the dose is large enough to display robust amnesic effects, at which point the representation becomes more elemental.

Saline controls	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	26.57	26.57	629.24	
Elemental Expected	39.65	52.73	760.63	
Actual Values	30.61	37.86		< 0.001
1 mg/kg	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	34.21	34.21	530.4	
Elemental Expected	46.82	59.43	627.05	
Actual Values	34.62	46.89		< 0.001
5 mg/kg	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	24.27	24.27	432.89	
Elemental Expected	35.26	46.24	650.58	
Actual Values	25.94	28.78		< 0.001
10 mg/kg	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	12.62	12.62	517.77	
Elemental Expected	20.71	28.8	350.82	
Actual Values	21.64	29.57		< 0.001

Comparison of full training context memory by dose. A comparison of freezing responses across drug conditions for the '3 cues' group revealed a significant reduction in freezing for only the 10 mg/kg group compared to saline controls [$t(100) = 5.3$, $p < 0.001$].

Comparison of neutral context freezing by dose. A comparison of freezing responses across drug conditions for the '0 cues' group revealed no significant differences ($ts < 3.09$, $ps > 0.05$). This indicates that, on average, mice did not freeze differently to the neutral context across CPP dosages (including saline controls).

By cue type. A MANOVA with one within-subjects (cue type presence or absence) factor and one between-subjects (drug condition) factor revealed a significant main effect of cue type [$F(5,500) = 24.77$, $p < 0.001$]. There was a significant main effect of drug condition [$F(3,100) = 2.98$, $p = 0.04$] and a significant interaction between cue type and drug condition [$F(15,500) = 1.95$, $p = 0.02$].

Post hoc analysis revealed that freezing scores were significantly higher for partial contexts with the teepee present compared to partial contexts without the teepee ($t(100) = 7.53$, $p < 0.001$. However, the largest mean increase in freezing due to the teepee's presence was for the 10mg/kg group, where a 14.4% increase in freezing was attributed to the presence of the teepee in the partial contexts. Again, this is a small difference considering the average freezing to the neutral context for this drug group is 15%.

Overall, the saline controls did not significantly differ on freezing compared to any of the CPP dosage groups for the partial context tests (*ts* < 1.91, *ps* > 0.05). The significant main effect of drug condition arose from an overall difference in freezing to the partial contexts between the CPP 1mg/kg and CPP 10 mg/kg groups [$t(100) = 2.76$, $p = 0.03$].

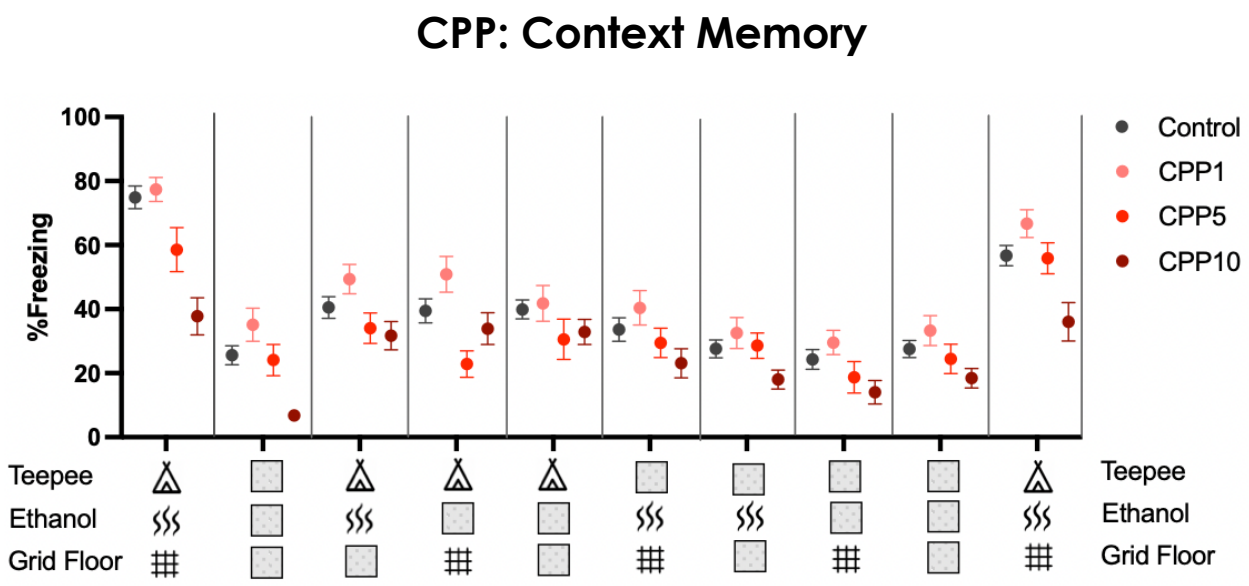

Figure 2.4: Comparison of the effects of a range of CPP doses given during training on long-term individual context tests. Symbols below each set of recent and remote points indicate the cues in each context configuration matching the training context; grey boxes indicate the absence of a given training cue. Error bars represent SEM.

CPP: Aggregated Cue Analyses

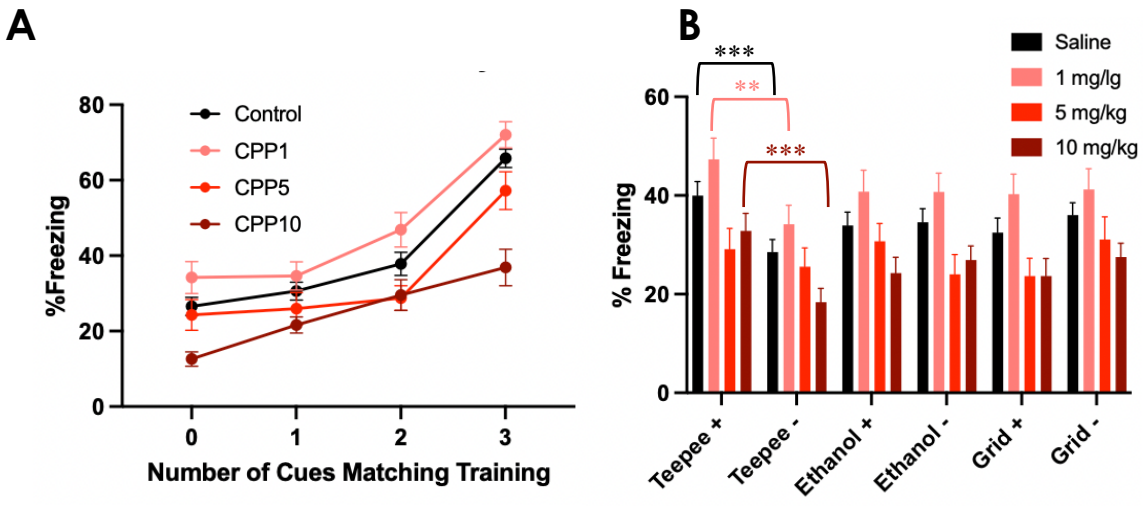

Figure 2.5: A) Aggregated mean context freezing data (from all ten context tests) based on the number of cues matching the original training context. See Table 2.5 for details on MANOVA post hoc analyses. **B)** Relative importance of cues from three sensory modalities: visuospatial, olfactory, and tactile. Aggregated mean freezing data grouped by the presence or absence of the three original training context cues (teepee/visuospatial, ethanol/olfactory, and grid/tactile). Freezing from only the six partial contexts is included. Data are depicted as means +/- SEM. Asterisks represent significant comparisons between the presence and absence groups for each cue type. For each comparison, * *Ps* < 0.05, ** *Ps* < 0.01, *** *Ps* < 0.001.

Esketamine

A MANOVA with one within-subjects factor (individual context test) and one between-subjects factor (drug condition) revealed a significant main effect of context test type [$F(9,846) = 76.96$, $p < 0.001$]. There was also a significant main effect of drug condition [$F(3,94) = 3.99$, $p = 0.01$] and a significant interaction between context test and drug condition[$F(27, 846) = 4.39$, $p < 0.001$]. Individual tests x drug condition:

Comparison against controls across all partial contexts. Post hoc comparisons were conducted for each partial context test comparing drug groups against the saline controls. However, no significant differences were found ($ts < 3.32$, $ps > 0.05$).

Number of Cues. A traditional analysis (MANOVA) was conducted first, revealing a significant main effect of number of cues matching the training context (within-subjects factor): [$F(3,282) = 232.75$, $p < 0.001$], a significant main effect of drug condition (between-subjects): [$F(3,94) = 4.18$, $p < 0.01$], and a significant interaction: [$F(9,282) = 5.15$, $p < 0.001$]. Post hoc analyses are featured in table 2.5. However, a more sensitive bootstrapping approach was employed to determine whether the data (aggregated based on the number of cues matching the training context) fit with a more elemental or more configural representation of the context by drug dose. Analysis of the esketamine data revealed a shift to an elemental representation for all dosages, regardless of amnesic effect (see Table 2.4).

Table 2.4: Goodness of Fit Comparison between custom configural and elemental models for contextual freezing data across doses of **esketamine** (including saline controls for reference). Mean squared errors (MSE), p-value, and observed vs. predicted freezing values for partial contexts with 1 and 2 cues matching the original training contexts are reported. Results of these analyses indicate that mice trained on esketamine represent the context elementally, even before an amnesic effect is readily observed.

Saline controls	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	26.57	26.57	629.24	
Elemental Expected	39.65	52.73	760.63	
Actual Values	30.61	37.86		< 0.001
1 mg/kg	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	33.52	33.52	788.74	
Elemental Expected	46.45	59.38	679.13	
Actual Values	44.39	56.15		< 0.001
5 mg/kg	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	21.18	21.18	1021.04	
Elemental Expected	36.48	51.79	801.65	
Actual Values	34.05	44.69		< 0.001
10 mg/kg	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	15.13	15.13	690.85	
Elemental Expected	26.12	37.11	446.49	
Actual Values	32.68	31.45		< 0.001

Comparison of full training context memory by dose. A comparison of freezing responses across drug conditions for the '3 cues' group revealed a significant reduction in freezing for only the 10 mg/kg group compared to saline controls [$t(94) = 3.66$, $p = 0.03$].

Comparison of neutral context freezing by dose. A comparison of freezing responses across drug conditions for the '0 cues' group revealed no significant differences ($ts < 2.82$, $ps > 0.05$). This indicates that, on average, mice did not freeze differently to the neutral context across esketamine dosages (including saline controls).

By cue type. A MANOVA with one within-subjects (cue type presence or absence) factor and one between-subjects (drug condition) factor revealed a significant main effect of cue type [$F(5,470) = 42.91$, $p < 0.001$]. Overall, regardless of dosage, freezing scores were significantly higher for partial contexts with the teepee present compared to partial contexts without the teepee [$t(94) = 7.53$, $p < 0.001$]. The presence of teepee yielded between 15% to 20% increase in freezing to the partial contexts across all esketamine dosages.

There was also a significant main effect of drug condition [$F(3,94) = 3.68$, $p = 0.02$]. The saline controls generally did not differ in freezing compared to any of the esketamine dosage groups for the partial context tests ($ts < 0.98$, $ps > 0.05$), except for the 1 mg/kg esketamine group, which yielded significantly higher freezing to the partial contexts overall compared to the saline group [$t(94) = 3.04$, $p = 0.02$].

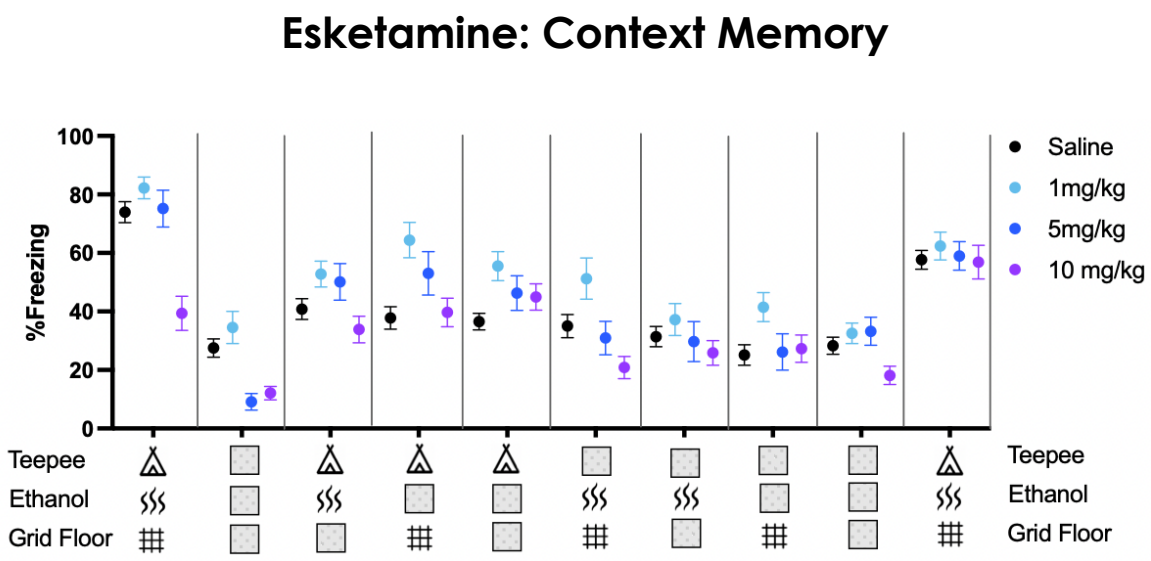

Figure 2.6: Comparison of the effects of a range of esketamine doses given during training on long-term individual context tests. Symbols below each set of recent and remote points indicate the cues in each context configuration matching the training context; grey boxes indicate the absence of a given training cue. Error bars represent SEM.

Esketamine: Aggregated Cue Analyses

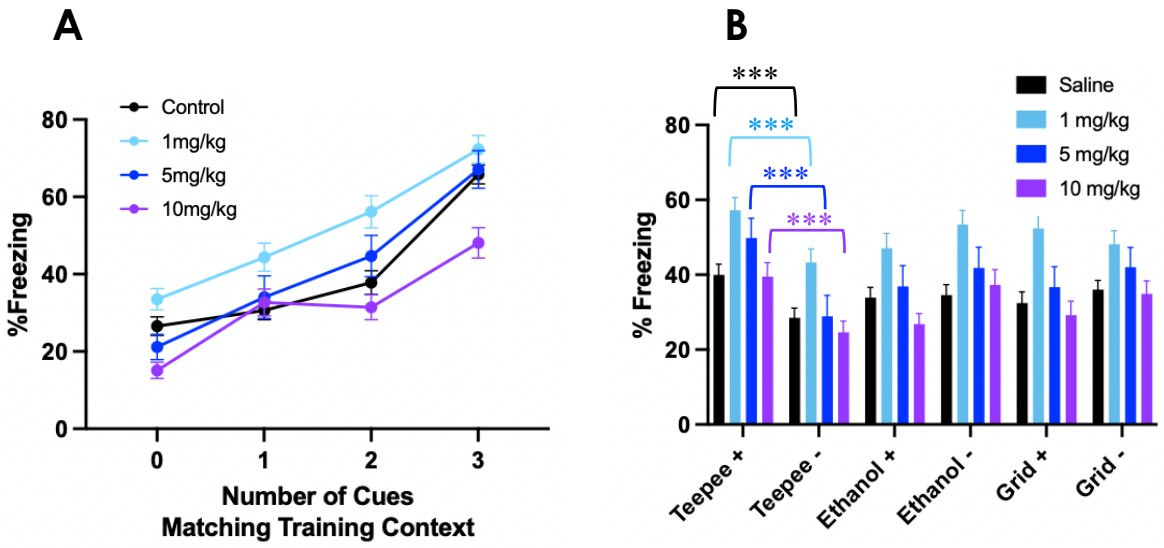

Figure 2.7: A) Aggregated mean context freezing data (from all ten context tests) based on the number of cues matching the original training context. See Table 2.5 for details on MANOVA post hoc analyses. **B)** Relative importance of cues from three sensory modalities: visuospatial, olfactory, and tactile. Aggregated mean freezing data grouped by the presence or absence of the three original training context cues (teepee/visuospatial, ethanol/olfactory, and grid/tactile). Freezing data from only the six partial contexts is included. Data are depicted as means +/- SEM. Asterisks represent significant comparisons between the presence and absence groups for each cue type. For each comparison, * *Ps* < 0.05, ** *Ps* < 0.01, *** *Ps* < 0.001.

Table 2.5: Supplemental post hoc analysis results from a MANOVA conducted to determine the effects of two factors [1) the number of cues matching the training context (within-subjects) and 2) drug condition (between-subjects)] on percent freezing. Asterisks indicate significant p-values.

Drug Condition		Group comparison (number of cues matching the training context)	t-statistic	p-value
Saline Controls		0-1	2.69	0.35
		1-2	4.11	< 0.01*
		2-3	16.23	<0.001*
Scopolamine	0.25 mg/kg	0-1	1.66	0.95
		1-2	3.19	0.12
		2-3	8.16	<0.001*
	1 mg/kg	0-1	0.62	0.99
		1-2	4.29	< 0.01*
		2-3	6.13	<0.001*
	5 mg/kg	0-1	2.99	0.19
		1-2	4.37	< 0.01*
		2-3	5.35	<0.001*
CPP	1 mg/kg	0-1	0.17	0.99
		1-2	4.2	<0.01*
		2-3	7.63	<0.001*
	5 mg/kg	0-1	0.67	0.99
		1-2	0.97	0.99
		2-3	8.62	<0.001*
	10 mg/kg	0-1	3.6	0.04*
		1-2	2.72	0.34
		2-3	2.22	0.68
Esketamine	1 mg/kg	0-1	3.46	0.06
		1-2	3.65	0.03*
		2-3	4.47	<0.01*
	5 mg/kg	0-1	4.1	<0.01*
		1-2	3.3	0.09
		2-3	6.2	<0.001*
	10 mg/kg	0-1	6.12	<0.001*
		1-2	0.42	0.99
		2-3	5.04	<0.001*

Discussion

The series of experiments detailed in this chapter explored how three pharmacological agents with amnesic properties (scopolamine, CPP, and esketamine) affect the representation of contextual fear memories in mice. Our findings reveal nuanced differences in how these drugs impact contextual learning and memory, providing insights into the mechanisms underlying contextual representations in the hippocampus. Consistent with Chapter 1 findings, intact mice (i.e., saline controls) demonstrated a configural representation of context, responding primarily to the full training context rather than individual elements.

Scopolamine, the muscarinic acetylcholine receptor antagonist, was most strongly predicted to produce a shift toward an elemental strategy by selectively impairing contextual memory, increasing generalization, and impairing perception (Anagnostaras et al., 1999; Pavesi et al., 2012). In examining memory of the training context, only 5 mg/kg of pre-training scopolamine produced significant amnesia, with 1 mg/kg producing a nonsignificant impairment and 0.25 mg/kg producing no impairment. However, when comparing the overall pattern of responding in the goodness of fit analysis (see Table 2.2), we see a strong configural strategy evident in saline controls, 0.25 mg/kg showing no preference for an elemental or configural pattern, and 1 mg/kg and 5 mg/kg producing a clear elemental pattern. Thus, for

scopolamine, the shift in response pattern from configural to elemental begins well below the dose that produces significant amnesia to the training context.

The NMDA receptor antagonist CPP was expected to produce a broad impairment in conditioning, as pre-training CPP produces a relatively equal impairment in context and tone conditioning (Carmack et al., 2013). As such, we expected both configural and elemental responding to be equally impaired, showing no particular tendency toward one model over the other in the goodness of fit test. However, CPP produced a significant impairment in memory to the training context only at 10 mg/kg. Consistently, CPP only produced a shift toward the elemental pattern of responding at 10 mg/kg. This suggests that the configural strategy is more sensitive to NMDA receptor antagonism than the elemental strategy.

Finally, esketamine, an allosteric NMDA receptor antagonist, produced a deficit in memory for the training context only at 10 mg/kg. However, similar to scopolamine, when examining the pattern of responding to partial cues, an elemental strategy emerges as dominant at 1, 5, and 10 mg/kg. These data suggest that the contextual deconstruction paradigm is more sensitive in detecting a shift from the configural strategy than the standard training context retrieval test, as a shift toward an elemental strategy was picked up at much lower doses for both scopolamine and esketamine.

Scopolamine and esketamine, despite having different mechanisms of action, both induced a shift to elemental processing before significant memory

impairment was observed. In contrast, CPP, which overlaps in mechanism with esketamine (NMDA receptor antagonism), maintained configural processing even as contextual memory began to decline. This dissociation suggests that the shift between configural and elemental context representation may be driven by higher-level cognitive processes rather than direct neural mechanisms.

Overall, the contextual deconstruction paradigm suggests a shift toward an elemental response pattern occurs under the influence of (at least these three) amnesic drugs during the acquisition phase of memory processing. This suggests that an elemental representation of context is relatively more resistant to cholinergic and NMDA receptor blockade compared to the configural representation. This is further consistent with the idea that all of these amnesic agents disrupt hippocampal function and cause a shift toward other memory structures that may underlie elemental responding. However, it is also clear that in intact mice this strategy is normally suppressed.

Acknowledgments

Chapter 2 includes material that is currently being prepared for submission for publication: Van Alstyne, K. & Anagnostaras, S. (in prep). The dissertation author was the primary researcher and author of this material.

CHAPTER THREE

The setting and the seahorse: the role of the dorsal hippocampus in representing contextual fear memories

Contextual fear conditioning, in which an animal comes to fear an environmental context that has been paired with shock, has become a leading model of declarative memory, as post-training lesions of the hippocampus produce a severe but time-limited retrograde amnesia selective to contextual fear (Kim & Fanselow, 1992; Maren et al., 1997; Anagnostaras et al., 1999; Anagnostaras et al., 2001). The hippocampus is thought to play a role similar to that in spatial memory, whereby it assembles a representation of the environmental context (akin to configural learning) that can then be associated with the US (i.e., footshock).

Despite this, several studies have found that pre-training lesions produce only a mild or nonexistent anterograde amnesia (Phillips & Ledoux, 1992; Maren et al., 1997; Frankland et al., 1998). This has led to speculation that in the animal with a damaged hippocampus, an alternative learning strategy must develop; this has been deemed the "elemental strategy" but this strategy must be suppressed when the hippocampus is intact (Anagnostaras et al. 2001). Despite wide acceptance of this view, it has yet to be directly tested whether intact or hippocampal-lesioned animals learn about the static elemental constituent components of the context, such as the visuospatial, odor, or grid floor cues.

In Chapters 1 and 2, using a novel context deconstruction paradigm, we found that intact animals do seem to use a configural strategy, robustly fearing the context but largely ignoring any of its constituent elements presented individually. This was largely true even into the consolidation period where memory becomes hippocampus-independent (Anagnostaras et al., 1999; 2001). In Chapter 2, we also found that drugs thought to impair hippocampal function, such as anticholinergics and NMDA receptor antagonists, caused a shift toward an elemental strategy, often at doses below those that cause evident amnesia to the training context. In this chapter, we will directly examine the effect of pre-training hippocampal lesions in our context deconstruction paradigm.

We begin our investigation with electrolytic lesions of the dorsal hippocampus, as most studies examining amnesia in this task used these lesions (Phillips & LeDoux, 1992; 1994; Kim & Fanselow, 1992; Frankland et al., 1998; Anagnostaras et al., 1999; Anagnostaras et al., 2000). We specifically chose lesion parameters from Frankland et al. (1998), as they found no deficit in context acquisition but found a deficit in discriminating two similar contexts in mice. Alternative approaches, such as using excitotoxic lesions were not chosen as a starting point because those lesions produce a much more extensive retrograde gradient, and produce deficits in cued fear as well, suggesting they may produce distal damage outside of the hippocampus (Anagnostaras et al., 2002).

Our study focused on lesions in the dorsal hippocampus, rather than the entire hippocampus, due to the functional distinction between dorsal and ventral regions (Fanselow and Dong, 2010). The dorsal hippocampus is primarily associated with spatial learning and memory, while the ventral hippocampus is involved in emotional processing and stress response (Moser and Moser, 1998; Kjelstrup et al., 2008; Kheirbek et al., 2013; Strange et al., 2014). Our primary interest is in contextual memory, which appears to be closely related to spatial mapping (O'Keefe and Nadel, 1978), a function primarily attributed to the dorsal hippocampus. Lesions in the ventral hippocampus have been shown to impair fear expression, particularly freezing behavior (Kjelstrup et al., 2002; Sierra-Mercado et al., 2011). Since our analysis relies on observing freezing responses, maintaining the integrity of the ventral hippocampus was crucial for our experimental design. By focusing on the dorsal region, we aim to specifically investigate the hippocampus' role in contextual memory while preserving the animals' ability to exhibit fear responses necessary for our analyses.

In line with the dual-representation view of contextual processing (Rudy et al., 2004; Harris, 2006; Rudy, 2009), we expect to see evidence for an elemental representation of the context in lesioned mice. As an elemental representation is proposed to focus on individual elements of the context, we expect to see an increase in freezing among the lesioned animals (compared to controls) to contexts presenting one or two training cues.

Materials and Methods

Animals

A total of 48 hybrid C57BL/6Jx129S1/SvImJ (129B6; Jackson Laboratory, West Sacramento, CA, USA) (24 males and 24 females) mice were used in total. This mouse strain was selected based on recommendations made at the Banbury Conference on Genetic Background in Mice, which was held to facilitate the comparison of results between experiments and among laboratories. This strain is often selected because of its strong performance on behavioral memory measures (Silva et al. 1997). Mice were weaned at 3 weeks of age and group-housed (2–5 mice per same-sex cage) with unrestricted access to food and water. The animal colony was maintained on a 14:10-h light/dark schedule, and all experimental procedures occurred during the light phase. Mice were at least 10 weeks old and handled for 3 days (1 min/day) prior to fear conditioning and testing. All animal care and experimental procedures were approved by the UCSD IACUC and compliant with the NRC Guide for the Care and Use of Laboratory Animals.

Fear Conditioning

The well-validated VideoFreeze software (Med-Associates Inc., Georgia, VT, USA) captured and scored each individual mouse's freezing behavior from an infrared camera (Anagnostaras et al. 2000, 2010) and controlled the tones and shocks to the chambers. Up to seven mice were trained and tested simultaneously in individual fear conditioning chambers (32 x 25 x 25 cm), which

consisted of stainless-steel sidewalls, white acrylic back walls, and clear polycarbonate front and top walls. Each chamber was transformed across three sensory dimensions to create distinct contexts (cues described below).

Context Cues

For all mice, all contexts consisted of three cues of three different sensory modalities (olfactory, visuo-spatial, and tactile). The full training context consisted of a strong 50% ethanol odor (olfactory), illuminated with a moderate (80 lux) white house light and triangular teepee (visuo-spatial), and a stainless-steel grid floor (tactile). The neutral context consisted of a weak (7% and rinsed with water) isopropyl alcohol odor (olfactory), no house light or teepee (visuo-spatial), and a white acrylic floor (tactile). Examples of the training and neutral contexts are depicted in Fig X. Prior to placing a mouse into the chamber, all surfaces within the space were cleaned with an odor matching the intended olfactory cue: either 50% ethanol or 7% isopropanol rinsed with water to minimize any residual odor, and wiped dry. Extra care was taken to ensure the strong ethanol odor did not linger in a chamber presentation intended to include the weak olfactory cue.

Experimental Procedures

Lesion procedure

Mice (30–50 g body weight) were anesthetized using isoflurane (USP, Fluriso, Vet One), which was administered through a vaporizer (Drager Vapor 2000, MFI Medical Inc., San Diego, CA, USA). A silicone tube connected the

vaporizer to a small, plastic, air-tight induction chamber (25cm x 16cm x 8cm). Anesthesia was induced rapidly (within about 30 seconds) by inhalation of a mixture of oxygen and 4% vaporized isoflurane. Mice were then transferred to a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), where they were fitted to a nose cone attached to a second tube with a reduced vaporization of 1.5-2% isoflurane. All exhaust was recovered by vacuum to a fume hood. This concentration of isoflurane provided sufficiently deep anesthesia, allowing surgery to be performed without signs of pain. Animals were randomly assigned to two groups: sham and dorsal hippocampal electrolytic lesion. For both sham and lesion procedures, the scalp was incised and retracted, and the mouse's head position was adjusted so that bregma and lambda skull landmarks appeared within the same horizontal plane. In addition, two small burr holes (about 2 mm in diameter) were drilled into the skull. This concluded the procedure for sham mice.

Lesioning procedures were similar to Frankland et al. (1988) and Anagnostaras et al. (2000), albeit with different anesthesia, because using these parameters pre-training in mice, they found no deficit in contextual fear acquisition but did find a deficit in context discrimination. For lesioned mice, stainless steel unipolar electrodes, insulated with plastic except for a 1mm tip, were inserted bilaterally into the dorsal hippocampus (-1.8mm posterior to bregma, ± 1.8 lateral to bregma, and 2mm deep), and attached to the anode of a DC stimulator with the negative terminal attached to the mouse's ear with

an alligator pad. Electrolytic lesions were made with anodal, constant direct current (3 mA for 5 sec) using a DC stimulator with an interval timer (World Precision Instruments, Sarasota, FL). Following surgical procedures, incisions for mice were closed with veterinary surgical adhesive (RiverBand formulated cyanoacrylate) and mice were given one dose intraperitoneally (i.p.) of 5 mg/kg meloxicam (Ostilox) to manage pain. Mice recovered from surgery in a clean, enclosed chamber heated at 25 degrees Celsius for approximately 30 minutes. Mice received six additional i.p. injections of 5 mg/kg Meloxicam (b.i.d. for three days) following surgery day. Mice were observed for five days after surgery for signs of abnormal behavior or health problems.

Fear Conditioning Training

Twelve to fourteen days after surgery, all mice underwent fear conditioning. Mice were placed inside the chamber with all the training cues (50% ethanol odor, house light and black teepee insert, and grid floor) present. The mice were recorded for 2 minutes prior to tone-shock pairings for baseline freezing and locomotor activity measures. The mice were given this pre-shock time period to allow them to adequately explore their environment, preventing the immediate shock deficit (Fanselow, 1986, 1990; Wiltgen et al., 2006; McHugh & Tonegawa, 2007). Following the baseline period, the mice experienced three tone-shock pairings. Each pairing consisted of a single 30s tone (2.8kHz, 85dBA) that co-terminated with a 2s scrambled foot shock (0.75 mA, RMS constant current, AC) that was transmitted through the grid floor. The interval between

pairings and after the final pairing was 30s. Thus, the mice were in the chamber for a total of 5 min during training. VideoFreeze software scored freezing and locomotor activity throughout the entire session.

Context testing

Beginning the day after training, all mice were tested (off-drug) on one context per day for ten consecutive days. On day one, all mice were tested on the neutral context, which is described above. On day two, all mice were tested on the original training context, also described above. The first two days consisted of baseline maximum responding to the training context and baseline minimum responding to the neutral context. On days 3-8, mice were tested on one of six partial contexts, where one or two cues matched the original training context, and the remaining cue(s) were from the neutral context. Partial contexts were counterbalanced over these six days. In order to evaluate the effects of repeated testing (for example, to determine if extinction occurred), two additional anchoring tests were completed on days 9 and 10. On the ninth day, all mice were retested on the neutral context, and on the tenth day all mice were retested on the full original training context. For each test, mice remained in the chamber for 5 minutes without experiencing any tones or shocks. Freezing was scored for 5 minutes to measure the cumulative effect of individual contextual cues on fear memory.

Staining procedure

Once testing was completed, all mice were sacrificed to determine the extent of dorsal hippocampal damage. They were deeply anaesthetized with 6% vaporized isoflurane and perfused with 4% paraformaldehyde in 0.9% saline transcardially. The brains were removed and placed in 4% paraformaldehyde kept at room temperature for 2 weeks. Brains were cryosectioned using dry ice and a sliding microtome. One-mm thick coronal sections (anterior to, posterior to, and through the cannula tracks) were made and then stained using thionin acetate according to standard procedures (i.e., with ethanol and xylene). The stained sections were mounted on slides and then covered.

Subject exclusion/inclusion criteria

A total of 23 mice received sham surgery, and 25 mice received dorsal hippocampal lesions. The sham group yielded ambiguous data with the goodness of fit analysis, showing not significantly better fit to either the elemental or the configural model. However, the sham data did not differ significantly from Chapter 2 saline controls across <u>all</u> freezing measures, and the saline and sham mice were run concurrently, thus the data were collapsed. In addition, two mice intended for the dorsal hippocampal lesion group were reassigned as shams, as no lesions were evident on either side of the brain after histological analysis. Thus, a total of 75 mice were included as controls in this experiment.

Of the remaining 23 mice intended for the dorsal hippocampal lesion group, one mouse died before histology, and samples from six other mice did not meet the criteria for acceptable lesions (primarily unilateral lesions), leaving 16 mice included in the dorsal hippocampal lesion group for statistical analyses. Inclusion criteria dictated that hippocampal lesions must have been made bilaterally, with damage extending through three cell layers (dentate, CA1, and CA3). Hippocampal damage was quantified using ImageJ software (Schneider et al., 2012). For slices through the lesion track (approx. 1.8 mm posterior to bregma), the total hippocampal area was manually defined and measured in square millimeters. Lesions were also manually outlined. We then calculated the lesion size as a percentage of the total hippocampal area. The percentage of left and right hippocampal damage was scored separately and averaged across both sides (weighted by hippocampal size) to determine the overall average damage (see Table 3.1). Animals with at least 20% overall average hippocampal damage were included in statistical analyses.

Table 3.1: Percentage of hippocampal damage estimated from a single plane (coronal slice; -1.82 mm posterior to bregma). Lesion extent is measured in square mm. All samples labeled 'included' came from mice included as part of the 'dh lesion' experimental group in all statistical analyses. Specific criteria for acceptable lesions are included in the methods section of this chapter. Two mice were reassigned to the control group, as histological analysis revealed no presence of a lesion on either side of the brain. All samples labeled 'excluded' were excluded from all statistical analyses. The notes column includes the reason for exclusion. Of included samples, the overall average of weighted total hippocampal damage was 48% (21-88% range).

Damage Left (%)	Damage Right (%)	Avg Total Damage (%)	Status	Description
0%	0%	0%	REASSIGNED	no lesion; reassigned to sham condition
0%	0%	0%	REASSIGNED	no lesion; reassigned to sham condition
71.9%	52.0%	62.2%	Included	Bilateral, extends through dentate, CA1, CA3
33.1%	26.7%	30.0%	Included	Bilateral, extends through dentate, CA1, CA3
34.5%	17.3%	26.1%	Included	Bilateral, extends through dentate, CA1, CA3
41.6%	25.2%	33.6%	Included	Bilateral, extends through dentate, CA1, CA3
11.8%	29.7%	20.6%	Included	Bilateral, extends through dentate, CA1, CA3
25.6%	27.2%	26.4%	Included	Bilateral, extends through dentate, CA1, CA3
29.3%	45.0%	37.0%	Included	Bilateral, extends through dentate, CA1, CA3
53.0%	41.1%	47.2%	Included	Bilateral, extends through dentate, CA1, CA3
94.7%	27.2%	61.7%	Included	Bilateral, extends through dentate, CA1, CA3
99.4%	47.0%	73.8%	Included	Bilateral, extends through dentate, CA1, CA3
25.6%	71.3%	47.9%	Included	Bilateral, extends through dentate, CA1, CA3
80.9%	94.5%	87.6%	Included	Bilateral, extends through dentate, CA1, CA3
90.4%	45.5%	68.5%	Included	Bilateral, extends through dentate, CA1, CA3
68.1%	59.4%	63.9%	Included	Bilateral, extends through dentate, CA1, CA3
35.4%	52.0%	43.5%	Included	Bilateral, extends through dentate, CA1, CA3
40.2%	37.6%	38.9%	Included	Bilateral, extends through dentate, CA1, CA3
0.0%	12.9%	6.3%	Excluded	unilateral and small
0.0%	42.1%	20.6%	Excluded	unilateral, but large
35.5%	0.0%	18.1%	Excluded	unilateral, but large
0.0%	57.4%	28.1%	Excluded	unilateral, but large
50.2%	0.0%	25.6%	Excluded	unilateral, but large
0.0%	60.9%	29.8%	Excluded	unilateral but large

Statistical Analyses

Univariate ANOVAs were conducted to measure differences in baseline locomotor activity and shock reactivity (measured in arbitrary units, Au; see Anagnostaras et al., 2010 for details) across drug condition.

As in Chapters 1 and 2, mean differences in context-freezing data for this set of experiments were analyzed using multivariate analyses of variance (MANOVAs) with one or two within-subjects factors and one between-subjects factor (surgery condition). In each of these analyses, percent freezing served as the dependent measure. Data from male and female mice were collapsed, as no statistically significant differences were found based on sex ($ps > 0.05$). Data from Chapter 2 saline controls and the lesion experiment sham subjects were merged, as no statistically significant differences were found between the two groups across individual context tests, number of training cue analyses, or cue type analyses ($ps > 0.05$). Post hoc comparisons were performed following significant group differences using Tukey's Honest Significant Difference (HSD) test. All data are presented as means ± standard error the mean (SEM). ANOVAs/MANOVAs and post-hoc analyses were conducted in Jamovi (version 2.5), an open-source statistical analysis software. Graphical representations of the data were created using Graphpad Prism 9 (GraphPad Software, LLC).

To compare the fit of each condition's (i.e., shams, lesioned mice) data to custom configural and elemental models of contextual fear conditioning, a permutation test focusing on the predictive accuracy for intermediate cue

conditions (1 and 2 matching contextual cues) was employed. These analyses were performed using R (version 4.2; R Core Team, 2024) with the dplyr package. See Chapter 1 for full details on this method of analysis.

Results

Baseline locomotion

To determine if dorsal hippocampal lesions affected baseline locomotor activity differently than controls (saline and shams), an independent samples t test was conducted. The dependent variable was the average locomotor activity during the 2-minute baseline period on training day. This test was performed to rule out potential confounds in freezing data caused by high baseline activity. Results of the t-test indicate no significant statistical difference between the lesioned and control mice [$t(72) = 0.96$, $p = 0.34$].

Shock reactivity

Likewise, to determine whether the lesions affected shock reactivity (average activity during shock transmission) differently than controls, another independent samples t-test was conducted. This test was performed to rule out the possibility that the two experimental groups experienced differential magnitude in the aversive nature of the shock, which could confound the freezing data. The t-test indicated that there was no significant difference between lesioned mice and controls on shock reactivity [$t(72) = 0.41$, $p = 0.68$].

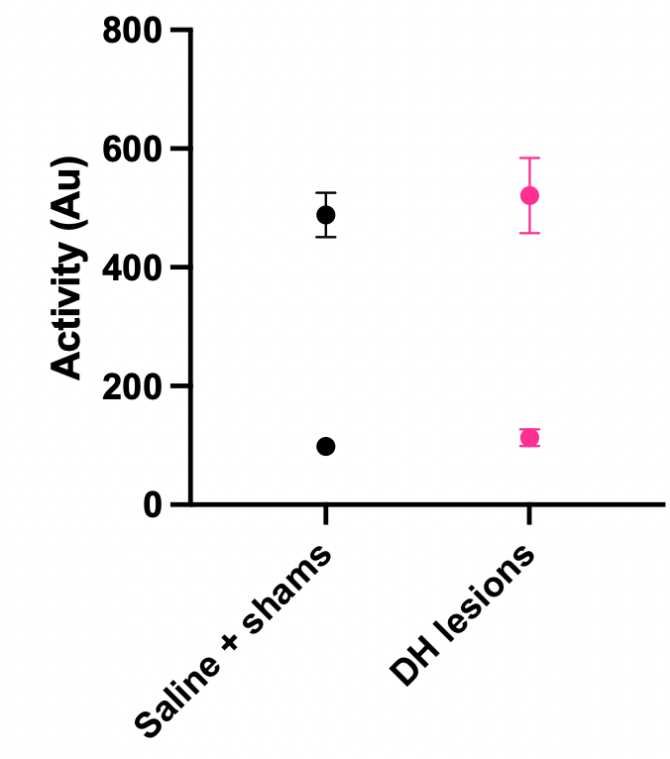

Figure 3.1: Baseline locomotor activity during the training day and average shock reactivity across all three training shocks were analyzed to identify potential confounds in the lesion-induced amnesic effect. This analysis was used to rule out alternative explanations for reduced freezing scores observed from context tests. There was no significant difference between the lesion group and controls on either baseline activity or shock reactivity measures.

DH lesions versus controls

Individual Tests. A MANOVA with one within-subjects factor (individual context test) and one between-subjects factor (surgery condition) revealed a significant main effect of context test type [$F(9,576) = 80.81$, $p < 0.001$]. There was no significant main effect of surgery condition [$F(1,64) = 1.13$, $p = 0.29$], suggesting that DH-lesioned mice and controls did not differ significantly on the amount of context freezing overall. There was a significant interaction between context test and drug condition [$F(9,576) = 2.57$, $p = 0.01$]. However, there were no differences found when controls and lesions were compared at each individual context test, including both training contexts and both neutral contexts, as well as all partial contexts ($ts < 2.3$, $ps > 0.05$).

Number of Cues. A traditional analysis (MANOVA) was conducted first, revealing a significant main effect of number of cues matching the training context (within-subjects factor): [$F(3, 267) = 247.37$, $p < 0.001$], but there was still no main effect of surgery condition [between-subjects factor; $F(1,89) = 1$, $p = 0.32$]. There was a significant interaction between number of training cues present and surgery condition [$F(3,267) = 4.59$, $p < 0.01$]. Post hoc analyses are detailed in Table 3.3, which appears to reveal a similar pattern of responding between both DH-lesioned and control mice.

Table 3.2: Supplemental post hoc analysis results from a MANOVA conducted to determine the effects of two factors [1) the number of cues matching the training context (within-subjects) and 2) condition (between-subjects)] on percent freezing. Asterisks indicate significant p-values.

Condition	Group comparison (number of cues matching the training context)	t-statistic	p-value
Controls (saline + shams)	0-1	4.25	< 0.01*
	1-2	5.54	< 0.001*
	2-3	15.99	<0.001*
DH lesions	0-1	4.28	< 0.01*
	1-2	2.88	0.09
	2-3	7.52	<0.001*

A comparison of freezing responses between DH-lesioned mice and shams for the '3 cues' group revealed no significant difference [$t(89) = 1.6$, $p = 0.72$]. A comparison of freezing responses between DH-lesioned and control mice for the '0 cues' group also revealed no significant difference [$t(89) = 0.61$, $p = 0.99$]. Together, these findings suggest both groups of mice learned the context equally well.

To more directly answer the question of whether DH-lesioned mice employ a more elemental or more configural representation of the context, a more sensitive bootstrapping approach was used to analyze this data set. Analysis of the data revealed a shift to an elemental representation in the DH-lesioned mice (see Table 3.2). The mixed results from the MANOVA and the permutation tests on the same data set suggests that the lesioned mice do respond more than controls to the individual cues, but they still show the ability to configure.

Table 3.3: Goodness of Fit Comparison between custom configural and elemental models for contextual freezing data between combined saline and sham **controls and lesioned** mice. Mean squared errors (MSE), p-value, and observed vs. predicted freezing values for partial contexts with 1 and 2 cues matching the original training contexts are reported. Results of these analyses indicate that mice with pre-training dorsal hippocampal lesions represent the context in a relatively more elemental (as opposed to configural) manner.

Saline + shams	1 Cue Group Freezing (%)	2 Cues Group Freezing (%)	MSE	P-value
Configural Expected	25.69	25.69	635.92	
Elemental Expected	39.04	52.4	748.72	
Actual Values	31.49	39.04		< 0.001
DH Lesions				
Configural Expected	22.94	22.94	851.25	
Elemental Expected	40.18	57.42	656.8	
Actual Values	35.57	47.46		< 0.001

By cue type. A MANOVA with one within-subjects (cue type presence or absence) factor and one between-subjects (surgery condition) factor revealed a significant main effect of cue type [$F(5,445) = 31.45$, $p < 0.001$]. Both DH-lesioned and control mice froze significantly more to partial contexts with the teepee present compared to partial contexts without the teepee [$ts > 6.44$, $ps < 0.001$]. Teepee vs no teepee for both groups was significant ($ts > 6.44$, $ps < 0.001$). On average, the controls froze 11.65% more to the partial contexts with the teepee compared to those without it, while the DH-lesioned mice froze 17.5% percent more to the partial contexts with the teepee. While the lesioned group froze more to the teepee's presence in the partial contexts on average

compared to the controls, the amount of freezing afforded due to the teepee between control and lesions did not differ significantly [$t(89) = 1.69$, $p > 0.05$].

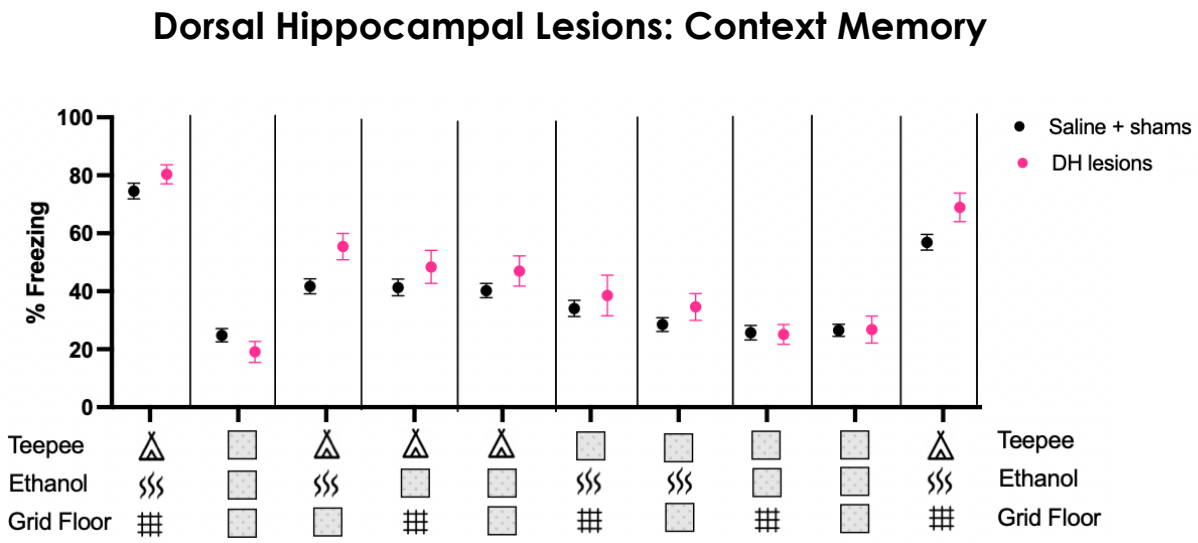

Figure 3.2: Effects of dorsal-hippocampal lesions on long-term individual context tests. Symbols below each set of recent and remote points indicate the cues in each context configuration matching the training context; grey boxes indicate the absence of a given training cue. Error bars represent SEM.

DH lesions and controls: Aggregated Cue Analyses

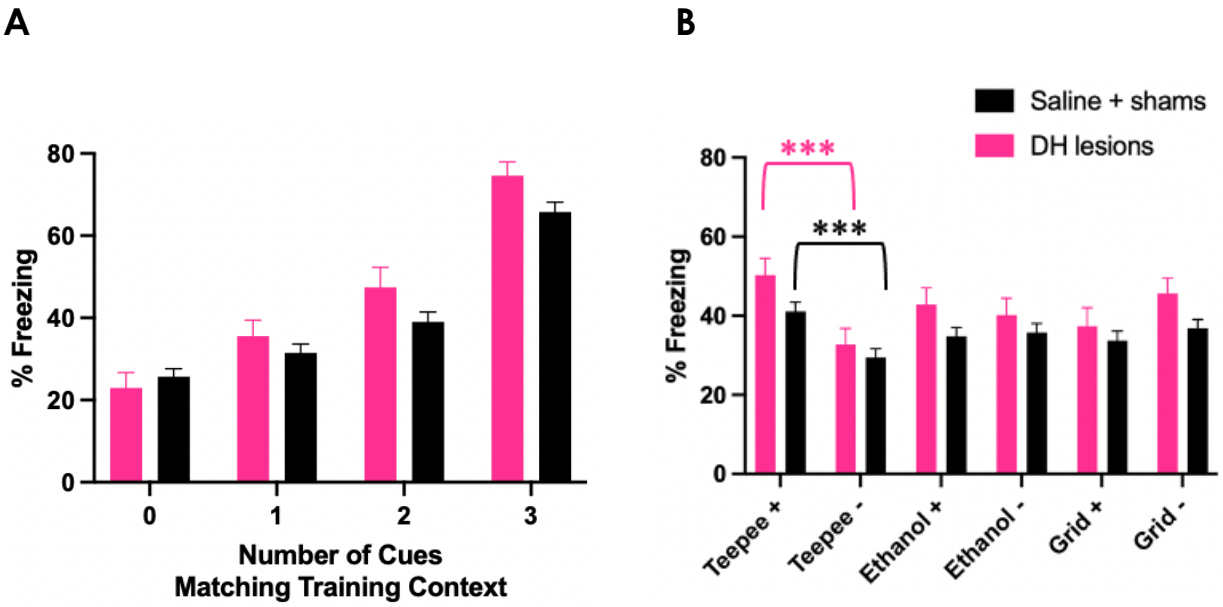

Figure 3.3: **A)** Aggregated mean context freezing data (from all ten context tests) based on the number of cues matching the original training context. See Table 3.3 for details on MANOVA post hoc analyses. **B)** Relative importance of cues from three sensory modalities: visuospatial, olfactory, and tactile. Aggregated mean freezing data grouped by the presence or absence of the three original training context cues (teepee/visuospatial, ethanol/olfactory, and grid/tactile). Freezing data from only the six partial contexts is included. Data are depicted as means +/- SEM. Asterisks represent significant comparisons between the presence and absence groups for each cue type. For each comparison, * *Ps* < 0.05, ** *Ps* < 0.01, *** *Ps* < 0.001.

Discussion

Our dorsal hippocampal (DH) lesion study provides new insights into the nature of contextual representations and the impact of hippocampal lesions on fear learning and memory. Contrary to some previous studies (Phillips & LeDoux, 1992; Kim et al., 1993), we did not observe a significant overall deficit in contextual fear conditioning in mice with DH lesions. Both lesioned and control mice demonstrated robust freezing to the full training context as well as similar levels of conditioning to the neutral context, confirming the findings that the dorsal hippocampus is not strictly necessary for the acquisition of contextual fear memories (Maren et al., 1997, 1998; Frankland et al., 1998; Gerlai, 1998; Cho et al., 1999; Wiltgen et al., 2006). Our finding of strong fear conditioning in the DH lesion group was also expected, given the robust training parameters with three tone-shock pairing used, and lesioned modeled after Frankland et al (1998).

Interestingly, both lesioned and control mice showed a preference for the visuospatial cue (teepee), freezing significantly more to partial contexts containing this element. This finding highlights the importance of visual information in contextual fear memories and suggests that some aspects of cue salience may be preserved even in the absence of normal hippocampal function. This is consistent with the idea that stimuli compete for associative strength during learning (Wagner et al., 1968; Rescorla and Wagner, 1972), and that more salient stimuli will overshadow less salient ones. The fact that this

overshadowing effect was observed in both lesioned and control mice suggests that it is not dependent on the hippocampus.

While the averaged freezing data across context test and number of matched training cues did not appear to be significantly different between the DH lesioned animals and controls, our more detailed analysis revealed subtle but important differences in how DH lesioned mice processed and responded to contextual information. The goodness of fit analysis indicated a shift towards a more elemental representation of context in lesioned mice, supporting the dual-representation theory of contextual processing (Anagnostaras et al., 2001; Rudy et al., 2004; Harris, 2006; Rudy, 2009). This shift suggests that while DH-lesioned mice can form contextual fear memories, they may rely more heavily on individual elements of the context rather than a unified, configural representation.

Our results also have implications for understanding the temporal dynamics of contextual fear memories. The fact that DH-lesioned mice could form and express contextual fear memories suggests that other brain regions, such as the amygdala, medial prefrontal cortex, or neocortical areas, may be sufficient for some aspects of contextual fear learning (Frankland et al., 2001; Frankland and Bontempi, 2005; Rudy, 2009; Honey et al., 2014; Maren et al., 2013). This finding is consistent with the systems consolidation theory, which proposes that memories become less dependent on the hippocampus over time (Squire & Alvarez, 1995; Frankland & Bontempi, 2005). However, the DH

lesioned mice showed a subtler shift towards an elemental strategy than expected. The amnesic drug dosage conditions from Chapter 2 exhibited a more pronounced linear trend in freezing behavior as training cues increased, compared to the DH lesioned mice. While the goodness of fit analysis indicated an elemental strategy in lesioned mice, they seemingly retained some ability to configure individual cues.

This observed difference between the amnesic drug dosage conditions and the lesioned mice may very well be due to the timing of the hippocampal manipulation relative to training. In contrast to lesions, when pre-training infusions into the hippocampus are made immediately before training with inactivating agents (Chang et al., 2008), anticholinergics (Gale et al., 2003), or NMDA receptor antagonists (Schenberg et al., 2008), they seem to produce robust anterograde amnesia, perhaps because animals have not had a recovery time period to develop an alternative strategy. Likewise, more extensive lesions, lesions made using other techniques, or those made under different training parameters, may yield a stronger shift toward an elemental strategy. These ideas need further exploration.

Together, our findings support a nuanced view of hippocampal involvement in contextual fear conditioning. While the dorsal hippocampus may not be strictly necessary for the formation of contextual fear memories, it appears to play a crucial role in shaping the nature of these representations. The shift from a configural to a more elemental strategy in DH lesioned mice

highlights the flexibility of fear learning systems and the potential for compensatory mechanisms in the face of hippocampal damage. These results contribute to our understanding of how the brain processes complex environmental information and forms emotional memories.

Acknowledgments

Chapter 3 includes material that is currently being prepared for submission for publication: Van Alstyne, K. & Anagnostaras, S. (in prep). The dissertation author was the primary researcher and author of this material.

GENERAL DISCUSSION

This dissertation work provides compelling evidence for the dominance of configural processing in contextual fear conditioning among intact animals, while also revealing nuanced shifts towards elemental processing under certain conditions. Our findings contribute to a deeper understanding of how mice represent and process contextual information in fear learning.

Configural Dominance in Intact Animals

Our results strongly demonstrated that intact mice predominantly acquire a configural representation when learning about fearful contexts. This was evidenced by robust conditioning to the full training context but minimal fear responses to individual contextual elements (i.e., partial presentations of the training context). This pattern held true across various experimental conditions, including signaled (tone-paired) and unsignaled (tone-absent) training protocols and for both recent and remote memories. These findings align with and extend previous theoretical propositions about the nature of contextual learning (Rudy et al., 2004; Anagnostaras et al., 2001), providing direct empirical support for the configural view of context processing.

Our results are consistent with the configural theory of contextual processing proposed by Rudy and Sutherland (1995), which posits that the hippocampus binds individual features into a unified contextual representation. The minimal importance of individual cues observed in our study suggests that once this configural representation is formed, the individual elements become

less salient. This finding may help explain why hippocampal lesions often result in global contextual memory deficits rather than specific impairments related to particular sensory modalities (Kim and Fanselow, 1992).

The stability of this configural representation over time and across repeated testing is particularly noteworthy. Unlike some previous studies that found increased generalization over time (Wiltgen & Silva, 2007), our results showed remarkable specificity in contextual fear memories, even after a 50-day delay. This stability suggests that the initial configural encoding of context may create a more robust and enduring memory trace than previously thought, at least in the hybrid mouse strain used in our studies.

Shifts Towards Elemental Processing

While configural processing dominated in intact animals, our experiments revealed several conditions under which a shift towards more elemental processing occurred. First, as memory aged, mice trained with unsignaled shocks showed a shift toward an elemental pattern ofresponding. Both scopolamine and esketamine administration led to a dose-dependent shift towards elemental processing, even at doses below those producing significant amnesic effects. This suggests that cholinergic and glutamatergic systems play crucial roles in supporting the development of configural representations of context. CPP administration maintained configural processing at lower doses but shifted towards elemental processing at higher, amnesic doses. The fact that CPP and esketamine share a similar primary mechanism of action (NMDA

receptor antagonism) yet displayed a slightly altered pattern of results could either be due to differing activity at other sites, or it could be due to a difference in higher-level cognitive processing (i.e., perception).

Dorsal hippocampal lesioned mice demonstrated a tendency to shift towards an elemental representation of context, though this change was not immediately evident from average freezing levels alone. While these findings support theories that the hippocampus is crucial for configural context representation, and its impairment results in elemental processing by alternative circuits (Rudy et al., 2004; Harris et al., 2006), our data suggest this transition is subtle. Notably, average freezing behavior appeared similar between controls and lesioned mice. This suggests that subjects with hippocampal damage may retain some ability to form configural representations, even if they no longer primarily rely on them. This could change with more extensive lesions, but previous studies have found that even mice with complete hippocampal lesions can acquire context conditioning with extensive training (Wiltgen et al., 2006).

Evidence from negative patterning experiments supports the idea that configural processing can occur without the hippocampus. In these experiments, two stimuli are reinforced when presented separately but not when presented together. This task is thought to require configural processing, as withholding responses to the compound stimulus implies the learner treats it as a unique configural entity (Rescorla and Wagner, 1972; Sutherland and Rudy, 1989). However, studies have shown that animals with hippocampal lesions can

learn negative patterning (Davidson et al., 1993; Richmond et al., 1997). Interestingly, just like with context memory, post-training hippocampal lesions initially disrupted negative patterning performance, but with retraining, animals reacquired the ability (Richmond et al., 1997).

Together, these findings suggest that while an intact hippocampus facilitates normal learning, other brain regions can compensate when it is damaged. These alternative circuits may support some degree of configural learning, particularly for less complex tasks.

Salience by cue modality

Freezing to individual contextual cues in intact mice was minimal and largely equivalent across different sensory modalities, with only a slight increase in freezing to the visuo-spatial cue (teepee). The slight preference for the visuo-spatial cue aligns with the well-established role of the hippocampus in spatial cognition and navigation (O'Keefe and Nadel, 1978; Moser et al., 2008). However, the overall similarity in responses to cues from different sensory modalities underscores the animal's capacity for multimodal integration in fear conditioning, as highlighted by Zhang and Manahan-Vaughan (2015). Their study demonstrated that in the absence of visual cues, rats can use olfactory information to generate stable place fields, indicating the flexibility of hippocampal sensory processing in forming spatial representations.

Furthermore, the observed minor preference for the visuo-spatial cue (teepee) over other contextual elements, which became more pronounced

over time, provides support for a hierarchical organization of spatial information in the hippocampus. This finding aligns with the framework proposed by Jeffery et al. (2004), who suggested that environmental layout (i.e., the shape of the space) and non-spatial contextual information (i.e., texture, odor) may be processed differently in the formation of spatial representations, where the layout was responsible for the fundamental framework of the contextual representation. More recently, researchers have found evidence for the neural basis of this hierarchy, as hippocampal place cells in rodents encode object, odor, and texture information in conjunction with spatial location, and this conjunctive coding develops in parallel with learning in contextual fear conditioning (Komorowski et al., 2009; O'Keefe and Krupic, 2021).

Our results extend this idea by demonstrating a temporal component to this hierarchy. Although multiple sensory modalities are available, visuo-spatial cues may take some precedence, especially as the representation consolidates over time. This shift may reflect a balance between maintaining a detailed representation of the environment and creating a more efficient, generalized spatial framework but a configural representation still prevails with memory age.

Repeated context testing and stable representations

The C57BL/6Jx129S1/SvImJ hybrid mouse strain used in this study demonstrated several characteristics that make it an excellent choice for future behavioral learning and memory research. This strain offers significant benefits in terms of memory stability, as evidenced by the performance of intact mice

across experiments within this dissertation. One of the most striking features of this hybrid strain is the remarkable stability of its contextual fear memories for an extended period of time (after two months, see Chapter 1) and this stability is perhaps comparable to rats which can show very stable memory even across their entire adult lifetime with extensive training (Gale et al., 2004). These mice maintained highly specific and robust contextual fear memories even after extensive testing. This long-lasting stability allows for extended experimental timelines, enabling researchers to study both recent and remote memory processes within the same subjects.

Utility of the context deconstruction protocol

The context deconstruction protocol introduced in this dissertation represents a significant advancement in fear conditioning methodologies, offering a more nuanced and comprehensive approach to understanding contextual learning and memory. This protocol's ability to provide substantially more data than standard fear conditioning paradigms with the same number of mice addresses a critical gap in the field, allowing for the detection of subtle differences that may have been obscured in previous studies.

Traditional fear conditioning protocols typically assess freezing behavior only in the full training context and a neutral context (e.g., Kim & Fanselow, 1992; Maren et al. 1997; Anagnostaras et al., 1999), which can mask important nuances in how animals process and remember contextual information. The context deconstruction protocol, by systematically manipulating individual

contextual cues, allowed us to dissect the relative contributions of cues from different sensory modalities to the overall contextual representation. Furthermore, it provided a means to assess the extent to which mice automatically process contextual cues as a configural unit.

The level of detail from this new protocol is particularly crucial when investigating the effects of manipulations such as dorsal hippocampal lesions, which do not readily show a deficit in the standard protocol that only measures memory to the full training context (Maren et al., 1997, 1998; Frankland et al., 1998; Gerlai, 1998; Cho et al., 1999; Wiltgen et al., 2006). This ability to detect nuanced changes in contextual representations opens new avenues for investigating the specific roles of brain regions and neural circuits in contextual learning and memory.

Limitations

The experiments detailed in this dissertation have a number of limitations. First, we used hybrid mice, and it may be the case that our findings are limited to mice (and not rats), or perhaps even to this strain of mice. Information gained from smaller dorsal hippocampus electrolytic lesions (modeled after Frankland et al., 1998) may not generalize to other approaches; they are intended to form a foundation by which other approaches (different or reversible lesions, etc.) can be examined. Finally, systemic scopolamine, CPP, and esketamine may not generalize to other ways of disrupting cholinergic or NMDA receptor function, or

to the effects of local infusion of these agents into the hippocampus (e.g., Gale et al., 2001).

In addition, the controls for the lesion experiment were collapsed with those from the drug experiments. The sham group yielded ambiguous data with the goodness of fit analysis, showing not significantly better fit to either the elemental or the configural model. However, the sham data did not differ significantly from Chapter 2 saline controls across all freezing measures, and the saline and sham mice were run concurrently. Thus, the data were collapsed. This is not an ideal solution, so we plan to run a replication of this experiment to achieve clarity on the differences between lesion and sham mice.

Future directions

As the context deconstruction protocol, in conjunction with a robust bootstrapping method, has demonstrated the ability to reveal even subtle differences to contextual representations in line with theoretical predictions (Rudy et al., 2004; Harris, 2006), this paradigm could be expanded in several directions. From the behavioral side, the equipment could be modified to make contextual variation easier and more flexible, such as making the elements re-arrangeable rather than just removable. From the neuroscience side, we intend to pursue this paradigm with more sophisticated approaches, for example disrupting local circuits in the hippocampus to dynamically direct animals from contextual to elemental strategies.

For researchers using fear conditioning to evaluate a drug's effects on learning and memory, this protocol offers some advantages. It allows for a more detailed characterization of how a drug might affect the processing of different contextual elements, potentially revealing effects that would be missed by standard protocols. The multiple test sessions also provide opportunities to assess how drug effects might change over time or with repeated testing, offering insights into the drug's impact on memory consolidation, retrieval, and extinction processes.

Furthermore, the protocol's sensitivity to subtle changes in contextual representations could be invaluable in studying models of neurological and psychiatric disorders that involve alterations in contextual processing, such as post-traumatic stress disorder (Van Rooji et al., 2015; Liberzon and Abelson, 2016) or drug addiction (Siegel, 1976; Badiani et al., 1995; Anagnostaras and Robinson, 1996; MacKillop and Lisman, 2008). By providing a more detailed picture of how contextual information is encoded and retrieved, this approach could lead to new insights into the neural mechanisms underlying these disorders and potentially inform the development of more targeted therapeutic interventions.

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