UNCOVERING THE ROLE OF THE RODENT DORSAL HIPPOCAMPUS IN SPATIAL AND OBJECT MEMORY RETRIEVAL

by

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ABSTRACT

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Male C7BL/6J mice were implanted with bilateral dorsal CA1 guide cannulae. After confirming that intrahippocampal microinfusion of muscimol impaired hippocampal function, demonstrated by impaired performance in the Morris water maze, the influence of intrahippocampal muscimol was tested in the Novel Object Recognition paradigm. During a test session 24 h after the last habituation/sample session, mice were presented with one familiar object and one novel object. Successful retention of object memory was inferred if mice spent more time exploring the novel object than the familiar object. Results demonstrate that muscimol infused into dorsal CA1 region prior to the test session eliminates novel object preference, indicating that the hippocampus is necessary for the retrieval of this non-spatial memory—a topic that has garnered much debate. Understanding the similarities between rodent and human hippocampal function could enable future animal studies to effectively answer questions about diseases and disorders affecting human learning and memory.

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PART I: INTRODUCTION

1.1 Purpose of studying rodent learning & memory

Insights into human learning and memory originated from observations of amnesic patients. These case studies provided an invaluable jump-start to the study of memory processes, but the information they provided was limited, to say the least. Fortunately, strong correlations can be drawn between humans, monkeys and rodents, thereby permitting more controllable studies. If human learning and memory, as well as the learning and memory deficits that result from injury or disease, can be modeled in non-human animals, it will be possible to conduct experiments that will vastly increase our understanding of the human brain. However, in order for this to be possible, one must first establish that specific species are similar enough to serve as animal models. The appropriateness of rodents as animal models for human learning and memory has encountered much debate. Confirming the similarities between rodent and human mechanisms of learning and memory will enable future research to utilize rodent models to address questions relevant to human learning and memory.

1.2 Multiple Memory Processes

Memory can be divided into several distinct processes. The initial acquisition of a memory is referred to as encoding. Consolidation is the process by which the memory is saved, enabling it to be recollected at a later time. Memories that are maintained over long periods of time are said to be stored. Retrieval is the memory process by which

previously consolidated and/or stored memories are brought into conscious recollection for application. Re-consolidation might comprise a final memory process: the idea behind re-encoding is that once a memory is retrieved, it must be re-consolidated if it is to be retrieved again at a later time. Whether or not specific types of memory are encoded, consolidated, stored and retrieved by one structure or not is a topic of much debate; alternatively, different structures might work in orchestration, each supporting different memory processes. This issue will be discussed in greater detail in later sections.

1.3 Multiple Memory Systems

It first became evident that the medial temporal lobe is the dwelling place of human memory in 1957 when Henry Gustav Molaison, the patient better known as H.M., underwent a medial temporal lobe resection in order to alleviate his debilitating drug-resistant epilepsy (Scoville & Milner, 1957). As a result of the operation, H.M.'s epilepsy was greatly alleviated; unfortunately, this remedy cost H.M. his memory. H.M.'s ensuing retrograde and anterograde amnesia provided evidence that structures within the medial temporal lobe are vital for memory (Scoville & Milner, 1957). However, H.M.'s otherwise intact intelligence and his unimpaired ability to perform day-to-day functions quickly clarified that memory is dissociable from other intellectual functions; furthermore, H.M.'s preserved ability to learn new motor skills and new perceptual skills demonstrated that memory comprises more than one entity and resides in more than one brain region (Corkin, 1984).

In addition to the studies of H.M., clinical studies of other patients supported the conclusion that the medial temporal lobe is necessary for some, but not all, types of learning and memory (Bertolucci et al., 2004; Rempel-Clower, Zola, Squire, & Amaral,

1996; Schmolck, Kensinger, Corkin, & Squire, 2002; Spiers, Maguire, & Burgess, 2001; Zola-Morgan, Squire, & Amaral, 1986) . In a 2001 comprehensive review, Spiers, Macguire and Burgess combined the findings of 147 case studies, as covered across 179 publications; they concluded that although bilateral hippocampal damage consistently resulted in severe episodic memory deficits, short-term memory, as is often assessed by asking the patient to immediately recall a span of digitally presented digits, and mnemonic, or implicit, abilities were preserved (Spiers et al., 2001).

Nonhuman primates with medial temporal lobe damage also demonstrate evidence of multiple memory systems; in fact, the theory of multiple memory systems was first generated from studies involving monkeys. In 1974, David Gaffan lesioned the fornix of monkeys, thereby damaging hippocampal – subcortical connections, and observed their performance in recognition and familiarity tasks relative to that of controls. His finding that recognition memory was impaired while associative, or familiarity-type, memory remained intact led to the understanding that only particular types of memory are dependent upon the hippocampus (Gaffan, 1974). Since that revelation, several studies have demonstrated that in spite of hippocampal damage, both monkeys and rodents are unimpaired at tasks assessing habit or skill learning (Squire, 1992).

While it is clear that the medial temporal lobe is necessary for some types of learning and memory in humans, monkeys and rodents, it is also evident that all three of these species exhibit types of memory that function independently of the medial temporal lobe. Thus, in addition to multiple memory processes, there are also multiple memory systems.

In a simplified model, memory can be subdivided into declarative and non-declarative categories (Figure 2). Declarative, also known as explicit, memory includes semantic memory for facts and episodic memory for events (Cohen & Eichenbaum, 2001); episodic memory also encompasses a strong spatial component. Declarative memory has been shown to rely on the medial temporal lobe. Non-declarative, or implicit, memory is memory for simple conditioning, procedures, motor skills and pattern analyzing, and seems to function relatively independently of the medial temporal lobe (Cohen & Squire, 1980; Davis, 2001; Squire, 1992; Squire & Zola-Morgan, 1988). Studies that take a closer look at the different types of memory and the structures that support them have made it possible to more strongly infer the similarities in learning and memory between humans, monkeys and rodents.

1.4 Implicit Memory

The independence of implicit memory from the medial temporal lobe is exemplified by H.M.'s ability to learn such perceptual-motor skills as mirror drawing despite his inability to remember learning such skills (Corkin, 1984). Likewise, patient Clive Wearing, an English musician who became amnesic after herpes encephalitis virus attacked his brain, is able to easily access the cups and sugar in his post-amnesia home even through he is unable, if asked, to remember where these things are located (Sacks, 2007). In addition to H.M. and Clive Wearing, clinical studies of other patients support the conclusion that medial temporal lobe damage does not directly affect implicit learning. For example, patient L.M., who developed amnesia after experiencing respiratory distress and several tonic clonic seizures possibly related to previous alcohol abuse, demonstrated impaired memory on both recall and recognition assessments for the

remainder of his life; he also suffered from impaired spatial location memory, but maintained normal implicit memory for spatial sequences and for skill learning (Rempel-Clower et al., 1996; Spiers et al., 2001).

The sparing of implicit memory despite medial temporal lobe damage has been documented in other species, as well (Squire, 1992). For example, monkeys who had undergone a bilateral resection of the hippocampi and amygdala performed as well as controls in a task that relied strictly on skill learning (Zola-Morgan & Squire, 1984), and rodents with medial temporal lobe lesions were unimpaired when learning simple passive avoidance tasks and tasks that required the learning of a single action in response to a sensory cue (Packard, Hirsh, & White, 1989). Likewise, Eichenbaum *et al.* (1988) found that rats with hippocampal lesions were able to outperform the control group in an odor discrimination task that required the rats to respond to successively presented odors in a go/no-go task. These findings provide evidence that rodents, monkeys and humans, alike, possess an implicit memory system that is distinct from other types of memory and independent from the medial temporal lobe.

1.5 Spatial Memory

On the other hand, it is well established that spatial memory depends on the medial temporal lobe (Eichenbaum, 2001). This subset of declarative memory supports learning and memory of the spatial environment and facilitates successful goal-oriented navigation.

Parslow *et al.* (2005) used an immersive virtual reality (IVR) system to assess allocentric spatial memory in patients who had undergone unilateral temporal lobectomies; they found that the right temporal lobe patients were impaired relative to

both controls and left temporal lobe patients, indicating the importance that the right temporal lobe plays in spatial memory (Parslow et al., 2005). However, whether human spatial memory depends uniquely on the right hippocampus or also on the right parahippocampal cortex has been debated. In a unique study, Veronique Bohbot and her colleagues assessed the abilities of patients who had endured right hippocampal and right parahippocampal cortical damage as a result of a single electrode thermocoagulation procedure attempted to alleviate epilepsy (Bohbot et al., 1998).Along with other groups, these patients were assessed in a battery of tests, many of which were intended to be human models of assessments typically used to assess rodents. Results from a Rey-Osterrieth complex figure task, in which patients attempt to redraw from memory a previously viewed picture, and an object location recollection task indicate that the right hippocampus is, indeed, necessary for some spatial tasks; however, the data from a test analogous to the Morris water maze, the invisible sensor task, indicate that damage restricted to the hippocampus results in some spatial memory savings, but that conjoint damage to the parahippocampal cortex causes more severe spatial impairments (Bohbot et al., 1998). The fact that the patients who comprised the right hippocampal lesion group performed unexpectedly well on several of the tasks might indicate that the lesions were not large enough to cause the extent of deficit that is generally associated with hippocampal damage; it is possible that the parahippocampal cortex group seemed more impaired not because the parahippocampal cortex is more important than the hippocampus in spatial memory but simply because the patients in the parahippocampal group generally had larger lesions which also encompassed a greater portion of the hippocampus.

One study that more strongly supports the specific role of the human hippocampus in spatial memory is a 1997 positron emission tomography (PET) study of London's taxicab drivers. The study revealed that the right hippocampus was preferentially activated when the drivers recalled routes through the city, though it was not activated when the same subjects recalled non-spatial information, such as plots from previously viewed movies (Maguire, Frackowiak, & Frith, 1997).

Studies of nonhuman primates further support the theory that spatial memory relies on the hippocampus. Parkinson, Murray and Mishkin (1988) found that cynomolgus monkeys who had undergone hippocampectomy performed at chance on both place recollection and object-place association tests, indicating that the hippocampus is necessary for spatial location memory whether or not it involves a more complex object-place association.

Rodents with hippocampal lesions also demonstrate a clear impairment on spatial tasks. Reports of the impairments resulting from hippocampal damage (Jarrard, 1978; Olton, Collison, & Werz, 1977) , combined with the discovery of place cells in the hippocampus of rodents (O'Keefe & Dostrovsky, 1971; Squire, Stark, & Clark, 2004) provided strong evidence that the hippocampus is necessary for spatial and contextual memory. Using single cell microelectrode recording, O'Keefe and Dostrovsky recorded the action potentials of hippocampal cells; they found that the firing of individual cells correlated with the rat's location in a testing arena, forming the foundation of Tolman's previously coined "cognitive map" (O'Keefe & Dostrovsky, 1971; Tolman, Ritchie, & Kalish, 1946). O'Keefe and Nadel's 1978 book, *The Hippocampus as a Cognitive Map,* bolstered the idea that the hippocampus plays an essential role in spatial memory

(O'Keefe & Nadel, 1978).

In 1982, Richard Morris published his findings on the spatial navigation abilities of rodents. He found that when placed in a pool of opaque water, later dubbed the Morris water maze, rodents could learn to efficiently navigate to a submerged platform (Figure 3). With enough training, the rats learned to use extra-maze visual cues to navigate to the platform despite the location of the starting point; in fact, well-trained rats were able to successfully navigate to the submerged platform even when they were placed in the pool from a novel starting location. Morris discovered that rats with hippocampal lesions were impaired at learning this spatial task when they had to rely on extra-maze cues to navigate, supporting the notion that spatial navigation is a hippocampal-dependent task (Morris, Garrud, Rawlins, & O'Keefe, 1982). The radial arm maze has also provided evidence that rodents with medial temporal lobe lesions are impaired on tasks that require the animal to remember where it has been (Packard et al., 1989), but the Morris water maze has become the standard rodent spatial navigation task. It has been used to demonstrate that, rodents, like humans and monkeys, possess spatial navigation memory that is dependent upon the hippocampus. Unfortunately, hippocampal contributions to the other domains of declarative memory are not as well understood.

1.6 Semantic Memory

Semantic memory, memory for facts that are not inextricably linked to specific events or contexts, raises the first question about declarative memory: is semantic memory, like episodic memory, housed in the hippocampus? Generally, episodic and semantic memory impairment go hand-in-hand, indicating that they are part of one memory system and both depend on the hippocampus (Squire et al., 2004); however,

clinical studies provide ample debate regarding this conclusion. Patients, such as H.M. and Clive Wearing, who clearly lack the ability to form new episodic memories, exhibit some preservation of semantic abilities. For example, in her 1984 report, Corkin stated that H.M. "…has islands of remembering, such as knowing that an astronaut is someone who travels in outer space, that a public figure named Kennedy was assassinated, and that rock music is 'that new kind of music we have'" (Corkin, 1984, p. 255). These 'islands of remembering' all represent semantic memories that H.M. could have only learned post-operatively. Likewise, Clive Wearing doesn't recognize the name of England's former Prime Minister John Major, yet Wearing spontaneously generates the name John Major when he sees the initials J.M. (Sacks, 2007).

As would be expected, bilateral hippocampal damage sustained early in life results in severe episodic memory impairment, but the intact semantic abilities and relatively normal cognitive development of these patients have been presented as evidence that semantic memory processes depend on neural substrates independent of the hippocampus (Vargha-Khadem et al., 1997). Vargha-Khadem *et al.* (1997) propose that semantic memory can be largely supported by the perirhinal and entorhinal cortices (Figure 4), and can therefore be sustained in spite of hippocampal damage. They argue that semantic memory is only affected in hippocampal patients when these underlying cortices are also affected, as in the case of H.M. (Scoville & Milner, 1957; Vargha-Khadem et al., 1997). This explanation that semantic memory impairment accompanies episodic memory impairment only in the presence of perirhinal and entorhinal cortical damage might provide an explanation for the variable impairments among amnesic patients (Spiers et al., 2001; Squire, 1992). Alternatively, it is possible that the patients in

the Vargha-Khadem study exhibited preserved semantic abilities because their hippocampal damage occurred so early in life that their brains developed compensatory mechanisms to support semantic learning (Squire et al., 2004). Of course, then one would wonder why compensatory mechanisms for episodic memory didn't also develop.

A 2003 study contradicted Vargha-Khadem *et al.*'s conclusion that semantic deficits ensue only when damage extends beyond the hippocampus, into the perirhinal and entorhinal cortices. Manns, Hopkins and Squire (2003) examined semantic knowledge deficits in five amnesic patients with damage limited to the hippocampus. They found that these patients did, indeed, exhibit significant semantic memory impairments. The results support the conclusion that semantic memory, like episodic memory, is encoded in the hippocampus. Alternatively, it is possible that the semantic impairments might not be a result of a specific semantic impairment, but rather a result of losing the aid episodic memories when trying to retrieving semantic facts; however, the study revealed that semantic memory is impaired in amnesic patients relative to controls even after controlling for the contribution of episodic memory when non-amnesic patients recall semantic memories. These results indicate that the semantic deficits observed in amnesic patients are not merely a result of losing the aid of episodic memory when retrieving semantic memory (Manns et al., 2003).

One possible explanation for the partially preserved semantic abilities of both early-onset and adult-onset amnesic patients is that the semantic memories are, by definition, not tied to a distinct episode; rather, they are general knowledge facts that are acquired after significant repetition. It makes sense that facts that are repeatedly encountered could eventually be learned, even if semantic memory *is* usually dependent

upon the hippocampus (Manns et al., 2003; Squire et al., 2004). Likewise, it would be expected that if these patients had repeated exposure to the same episodic event, it, too, would be remembered to the same handicapped degree that semantic abilities are preserved.

Despite these theories, it has been difficult to ascertain what accounts for an apparent partial saving of semantic memory in amnesic patients because of the challenge in completely separating semantic knowledge from episodic memories. Perhaps the semantic elements that are preserved in hippocampal patients reflect the effect of a hippocampal-independent familiarity-based form of memory. This raises another topic of debate in the literature regarding the role of the hippocampus in declarative memory; the debate regarding familiarity and recollection is discussed in Section 1.8.2.

1.7 Episodic Memory

Episodic memory, memory for the *what*, *when* and *where* details of events (Tulving, 2002), is the final subgroup of declarative memory to be discussed. Although episodic memory still presents some questions, one thing is clear: the human hippocampus plays an important role in episodic memory.

Across the board, patients who suffer hippocampal damage exhibit episodic memory deficits. Their abilities to encode new memories (anterograde) and, to varying degrees, to retrieve old memories (retrograde) are markedly impaired (Corkin, 1984; Spiers et al., 2001; Squire et al., 2004). The deficit that patients with hippocampal damage exhibit in forming and retrieving memories of personal experiences is strong evidence that this ability relies on the function of the hippocampus. Although not as clearly episodic in the traditional sense, episodic memory in patients is typically assessed

via recollection of verbally presented word lists or complex visual figures (Spiers et al., 2001; Vargha-Khadem et al., 1997).

Monkeys with surgically- or ischemia-induced hippocampal lesions also suffer from episodic memory deficits, as exhibited by impaired performance in delayed non-match to sample (DNMS) and delayed match to sample (DMS) tasks. In the DNMS and DMS tasks, the animal is rewarded for selecting a novel object over a familiar object, or a familiar object over a novel object, respectively, thereby demonstrating recognition of the familiar object as such (Squire, 1992).

Likewise, Eichenbaum *et al.* (1988) found that rats with hippocampal lesions were impaired in a go-left/go-right task that required them to alter behavioral choices based on the location of two different odors (Eichenbaum et al., 1988). Despite these findings, the role of the rodent hippocampus in episodic memory continues to be debated; a more thorough discussion of episodic memory as it relates to rodents is presented in Section 1.9.

1.8 Episodic Memory Debates

Clinical studies of patients who have suffered hippocampal damage and monkey and rat studies that induce hippocampal lesions implicate the hippocampus as an essential structure for declarative memory. However, several questions specific to declarative memory remain.

1.8.1 Does the hippocampus play a time-limited role in declarative memory?

The first question addresses the variability in retrograde amnesia, as presented in the Spiers *et al.* (2001) review. Although initial judgments estimated that H.M.'s retrograde memory dated back to about two years before his surgery, more in depth

interviews revealed that H.M. had actually lost his declarative memories dating back as far as eleven years before his medial temporal lobe resection (Corkin, 1984). The issue of H.M.'s retrograde amnesia is confounded by his history of epilepsy: eleven years before surgery coincides with the onset of H.M.'s epilepsy and his being treated with toxic doses of anticonvulsant medications (Corkin, 1984). Therefore, it is difficult to establish if H.M.'s retrograde amnesia was actually a result of his surgery or if it can be attributed to seizures or to the anticonvulsant medications with which he was treated for years. Less complicated examples of retrograde amnesia resulting from isolated hippocampal formation damage are those of patients L.M. and W.H. whose memory deficits dated back approximately 15 and 25 years, respectively (Rempel-Clower et al., 1996). Even damage limited to the CA1 field of the hippocampus appears sufficient to cause retrograde amnesia; patients R.B. and G.D. with such restricted hippocampal damage exhibited retrograde amnesia encompassing 1 to 2 years (Rempel-Clower et al., 1996). Due to the graded retrograde amnesia among hippocampal-damaged patients, it is widely believed that the hippocampus plays a time-limited role in the formation and storage of both semantic and episodic knowledge (Manns et al., 2003; Squire et al., 2004).

Aside from the variability in retrograde amnesia among hippocampal patients, one fact is clear: not all memories from the past are lost. It seems, then, that memories eventually become independent of the hippocampus. There are several theories as to how this happens. Traditional consolidation theory (TCT) posits that declarative memories are encoded in the hippocampus but are eventually transferred out to neocortical regions for long-term storage. This theory offers a logical explanation for the phenomenon of graded retrograde amnesia: patients suffering hippocampal damage are able to remember events

that were transferred to the intact neocortical regions prior to the onset of amnesia (Alvarez & Squire, 1994; Rempel-Clower et al., 1996). However, this theory is problematic because some patients with retrograde deficits might have "lost" all memories from the past two or three decades; traditional consolidation theory would imply that it must take this long for memories to be consolidated into the neocortex—a proposal that does not seem evolutionarily logical (Kopelman & Kapur, 2001). Multiple trace theory (MTT), on the other hand, presents the idea that memories are never shifted completely out of the hippocampal region; instead, with a first encounter, a memory trace between the hippocampus and the neocortex is created. Each time that memory is activated, the trace network grows bigger and more elaborate. Eventually, this network might be sustainable even in the event of hippocampal damage because of the strength of the trace (Nadel & Moscovitch, 1997). This theory is weakened, however, by patients with severe anterograde amnesia, indicating significant hippocampal loss, but retrograde amnesia expanding only two to three years back (Kopelman & Kapur, 2001); according to MTT, hippocampal damage widespread enough to cause severe anterograde amnesia should also result in severe retrograde amnesia. Also, MTT would predict an incredible network of traces for every retained memory; perhaps this volume of traces is not practical. Finally, a theory proposed by Cermak (1984) states that frequent rehearsal of episodic memories gradually makes them semantic. This theory would imply that over time memories become less vivid but better preserved; however, this possible explanation has two problems. First, it rests on the unconfirmed theory that semantic memory resides outside the hippocampus; second, it does not account for graded retrograde amnesia of semantic memories (Kopelman & Kapur, 2001). Despite these theories, the actual role of

the hippocampus in the retrieval of long-term memories remains unclear.

1.8.2 Are familiarity & recollection both supported by the hippocampus?

Eichenbaum classifies the distinction between familiarity and recollection; the former is a rapidly accessed "sense" that depends on the perirhinal cortex but not the hippocampus, while the latter involves qualitative associations and relies on the hippocampus and the parahippocampal cortex (Eichenbaum, Yonelinas, & Ranganath, 2007). Receiver operating characteristics (ROCs) are one method of visualizing this distinction: by plotting the proportion of hits to false alarms over varying confidence levels, one can produce a ROC curve whose y-intercept provides a measure of recollection and whose degree of curvilinearity provides a measure of the difference of familiarity between new and previously encountered items (Eichenbaum et al., 2007). Additionally, the remember/know distinction provides another way of delineating the difference between recollection, or remembering and familiarity, or knowing. The remember/know method requires subjects to distinguish between known items for which they can or cannot remember qualitative details (Eichenbaum et al., 2007). Similarly, the relational-recognition method also distinguishes between recollection in which subjects can remember the time or place when an item was studied and familiarity in which subjects can remember an item but cannot recall the details of where or when it was studied (Eichenbaum et al., 2007). Finally, event-related brain potentials (ERPs) recorded during memory tasks have revealed that familiarity depends more strongly on a mid-frontal region while activation in the parietal region is a more robust indication of recollection (Eichenbaum et al., 2007).

It was once believed that the medial temporal lobe (MTL) is necessary for

recollection but that familiarity is independent of the MTL and depends, instead, on the association cortex. More recently it has been hypothesized that the MTL is necessary for both recollection and familiarity (Eichenbaum et al., 2007), but this theory is undermined by the impairments found in amnesic patients with MTL damage: recollection is abolished, but familiarity is affected to varying degrees. These findings all seem to support the theory that the hippocampus is essential in supporting episodic-like recollection and that the parahippocampal region supports familiarity. In the presence of damage restricted solely to the hippocampus, familiarity is salvaged; however, when the parahippocampal region is also damaged then familiarity suffers, as well. Alternatively, Squire *et al.* (2004) argue that recollection and familiarity seem to be differentially affected by MTL damage not because they depend on different structures, but because they rely on different amounts of a functional hippocampus; in other words, while recollection might be affected with even 30% of hippocampal damage, more extensive damage (perhaps up to 75%) might be necessary before familiarity-based impairments are exhibited (Broadbent et al., 2004; Squire et al., 2004).

Despite the strides that have been made in understanding the role of the hippocampus, many questions remain regarding the memory systems of primates; even within the declarative memory category, there are subsets of memory that are difficult to attribute to a specific brain region. These questions, among others, also riddle the field of rodent studies.

1.9 The Rodent Hippocampus & Episodic Memory

The first challenge researchers face in studying declarative memory in rodents is establishing whether rodents even possess such a type of non-spatial memory (Cohen &

Eichenbaum, 2001). Ennaceur and Delacour took the first step in addressing this debate by developing a spontaneous novel object recognition (NOR) task for rats (Ennaceur & Delacour, 1988). The task was similar to the visual paired comparison (VPC) test (Fagan, 1970) that was previously used to assess novelty recognition abilities in human infants (Clark et al., 2000; Ennaceur & Delacour, 1988). In the novel object recognition task, the rat is first exposed to two identical objects during a sample session, which is followed by a brief delay and then the test session, in which the rat is exposed to two non-identical objects—one familiar object from the sample session and one novel object. Ennaceur found that rats will spend more time exploring the novel object than the familiar object (Ennaceur & Delacour, 1988), just as human subjects will look longer at a novel picture in the VPC task (Fagan, 1970). The rat's preference for the novel object, as indicated by significantly more time spent exploring it than spent exploring the familiar object, is evidence that the rat recognizes the familiar object from the sample session. This arguably non-spatial object memory is evidence that rodents do, indeed, possess a form of non-spatial episodic memory.

A great effort has been made to develop rodent tasks that require the same *what*, *when* and *where* recollections characteristic of human episodic memory. One such study implemented a modified version of an 8-arm radial maze in conjunction with a food-caching paradigm to demonstrate that rats are able to conjoin *what* memories (what food reward was presented) with *where* memories (which arm contained the different food rewards) and *when* memories (how long ago or what time of day the most preferential reward is available) (Babb & Crystal, 2006). These findings provide strong support that rodents possess episodic-like memory.

Other non-spatial rodent memory tasks have been developed to elicit the role of the rodent hippocampus in the specific components of episodic memory. *Where* features of episodic memory are exhibited in tasks that require spatial learning, such as the Morris water maze and the standard radial arm maze paradigm. *When* features are demonstrated in temporal tasks that require rats to demonstrate memory of a sequence of events, such as Fortin *et al*.'s study that rewarded rats for selecting the earlier presented odor when choosing between two odors from a previously presented sequence (Fortin, Agster, & Eichenbaum, 2002). Finally, the *what* component of episodic-like memory has been studied in rodents through matching tasks, like DNMS and DMS and through VPC/NOR tasks. In conjunction with hippocampal lesions, these paradigms are used in efforts to reveal whether the hippocampus is a necessary structure in rodent episodic-like memory. These studies tend to result in widely variable findings; for example, some DNMS studies found that hippocampal damage results in impaired performance (Clark, West, Zola, & Squire, 2001; Wood, Mumby, Pinel, & Phillips, 1993), while others found that it does not (Duva et al., 1997; Mumby et al., 1996; for a review, see Squire, Wixted, & Clark, 2007).

In 1999, Wood, Dudchenko and Eichenbaum tried a different approach: they surgically implanted dorsal hippocampal electrodes and conducted electrophysiological recordings while rats performed in a continuous non-match to sample task. Wood *et al.* modified the task so that rats were presented with an odor in any of 9 locations during the sample session and were then presented with an identical or different odor in another location. Rats were rewarded for digging on the non-match to sample (novel odor) trials. During the task, activity from complex spike cells in the CA1 and CA3 pyramidal cell fields was recorded. Wood *et al.* (1999) found that of the 127 single units recorded, 10

fired in response to specific odor stimuli (but not to trial type or location), 13 fired differentially for match or non-match trials (but did not vary among odors or location) and 26 fired specifically during the rat's approach or arrival at the stimulus cups (regardless of odor, trial type or location); 40.2% of the cells recorded represented non-spatial activity, while only 31.5% had even a partially spatial component (Wood et al., 1999). Wood *et al.* (1999) interpreted their findings as an indication that "non-spatial and spatial information are both fundamental components of hippocampal representations" (p. 615). This electrophysiological evidence combined with the multiple DNMS and NOR experiments that report object recognition deficits as a result of hippocampal inactivation provide strong support for the conclusion that rodents possess episodic-like memory and that, like primates, it is dependent upon the hippocampus

Despite these other techniques that seek to elucidate whether rodents posses non-spatial episodic memory, the NOR task has emerged as a principal tool in the debate. Countless studies have since utilized the NOR paradigm (interchangeable with VPC paradigm) in order to examine non-spatial object recognition memory in rodents. These studies, too, have frequently resulted in conflicting findings (Clark et al., 2000; Forwood, Winters, & Bussey, 2004; Squire et al., 2007).

Initial NOR studies indicated that rodents with hippocampal damage demonstrate NOR deficits (Clark et al., 2000; Wood & Phillips, 1991). Clark, Zola and Squire (2000) found that rats with radio-frequency or ibotenic acid lesions of the hippocampus exhibited delay dependent impairments relative to controls on a VPC task. The authors acknowledged that other studies report that perirhinal or entorhinal cortical lesions also result in visual recognition memory deficits, but they conclude that these findings, in

conjunction with their 2000 study, support the idea that recognition memory is "widely dependent on the structures of the medial temporal lobe memory system" (Clark et al., 2000, p. 8859).

Gaskin, Tremblay & Mumby (2003) found that cytotoxic lesions of the hippocampus impaired memory for objects explored prior to the surgery, but not for objects explored after the surgery. In a follow-up study, Mumby *et al.* (2005) reported that permanent hippocampal lesions caused by N-Methyl-D-aspartate, (NMDA), did not impair novel object preference with delays of 24 h, 1 week or 3 weeks. Mumby *et al.* (2005) interpreted their results along with the 2003 findings (Gaskin et al., 2003) to conclude that object recognition memory is usually encoded and stored by the hippocampus, so that if the hippocampus is compromised after the encoding of an object memory, that memory will be unavailable. However, they argue that in the absence of the hippocampus, extrahippocampal structures are able to support the encoding and storage of object representations (Mumby et al., 2005). This conclusion directly conflicts with that of a 2004 study in which Hammond, Tull and Stackman utilized intracranial microinfusions of lidocaine to temporarily deactivate the hippocampus of C57BL/6J mice prior to the encoding phase of the NOR task (Hammond et al., 2004). Hammond *et al.* (2004) found that interrupting hippocampal function during the encoding stage impaired object recognition when there was a 24 h delay between the sample (encoding) and test (retrieval) sessions, but not when there was only a 5 min delay. These results indicated that the hippocampus plays a delay-dependent role in object recognition memory, and is only necessary for longer delays; alternatively, the hippocampus might be involved even at short delays, but the parahippocampal structures might be sufficient to support the

memory when the hippocampus is unavailable at short, but not long delays (Hammond et al., 2004). The conclusions from the Hammond *et al.* (2004) study contradict the conclusion of Mumby *et al.* (2005) that when the hippocampus is unavailable during encoding the entorhinal and perirhinal cortices are able to support object recognition memory for long delays; if this was the case, then the mice that received intrahippocampal lidocaine microinfusions in the Hammond *et al.* study (2004) would not have demonstrated an impairment in the long-delay because the memory would have been supported by entorhinal and perirhinal cortices. The difference in findings between the Mumby *et al*. (2005) study and the Hammond *et al*. (2004) study might be a result of different degrees of hippocampal inactivation or of the experimental methods, such as testing procedures, arena differences, etc. Although unlikely, it is also possible that the permanent lesions implemented in the Mumby *et al.* (2005) study resulted in a reorganization of the structures that support memory; perhaps the parahippocampal and entorhinal cortices developed the ability to support long-term object recognition memory more than they do when the hippocampus is only transiently unavailable, as in the Hammond *et al.* (2004) study. This avoidance of plastic changes of memory structures is only one of the benefits of inducing temporary rather than permanent lesions in the hippocampus; other benefits of transient inactivation will be discussed later.

Forwood, Winters and Bussey (2004) hypothesized that impairments observed in NOR studies might be a result of a spatial or contextual component presented by the NOR paradigm; they modified the task by using a Y-shaped arena (Figure 3) rather than an open arena, and found that rats with ibotenic acid lesions of the hippocampus were unimpaired (Forwood et al., 2004). A concurring study that utilized the same modified Y-

shaped arena revealed evidence for a functional double dissociation of the hippocampus and perirhinal cortex: Winters *et al*. (2004) found that hippocampal lesions impaired performance on a radial arm maze of spatial memory but not on a novel object recognition test, while perirhinal lesions had the exact opposite effect. These findings indicated that the rodent perirhinal cortex—not the hippocampus—is involved in object recognition memory; it is a conclusion that has met significant opposition.

In a 2004 study, Broadbent, Squire and Clark continued to examine the effect of hippocampal lesions on visual recognition memory in rats; this time, they examined the relationship between lesion size and consequential impairment on both spatial and object recognition tasks (Broadbent et al., 2004). The finding that object recognition memory is impaired only with lesions that encompass at least 75% of hippocampal volume while spatial memory is impaired with lesions as small as 30-50% (Broadbent et al., 2004) provides one possible explanation for object recognition studies that did not find impairment with hippocampal lesions: perhaps only the largest hippocampal lesions result in object memory impairment.

In considering the vast number of variables between NOR studies—lesion size, lesion specificity, delay between sample and test, presence or absence of spatial or contextual components, and specific memory process(es) interrupted—it is no wonder that results from such studies present contradictory findings. A summary of some of these findings, along with the variables that might be responsible, is presented in Figure 1.

1.10 Hippocampal Circuits and the Dorsal CA1

One argument for the differential dependency of recollection and familiarity on the hippocampus is founded in the organization of the medial temporal lobe (Figure 4).

The medial temporal lobe can be roughly divided into the hippocampus, consisting of the dentate gyrus, Ammon's horn and the subiculum, and the parahippocampal region, consisting of the parahippocampal/postrhinal (in primates and rodents, respectively), perirhinal and entorhinal cortices. Information enters the medial temporal lobe in two basic ways: association areas that process unimodal sensory *what* information about objects is fed from association areas into the perirhinal cortex; this *what* information then travels to the lateral entorhinal cortex and then into the hippocampus. On the other hand, polymodal *where* information is directed into the parahippocampal cortex, then to the medial entorhinal cortex and then into the hippocampus. These *what* and *where* pathways converge in the hippocampus, which projects, among other places, back to the entorhinal cortex, then to the perirhinal and parahippocampal cortices and, finally, back to the neocortical regions from which the information originated. It has been argued that the representation of unimodal information maintained by the perirhinal and lateral entorhinal cortices and the feedback projections from these cortices to the neocortex might be sufficient to support familiarity-type memory. Recollection-type memory, on the other hand, requires the hippocampus because of the role that this structure plays in associating the various elements necessary for episodic-like recollection (Eichenbaum et al., 2007). Since the hippocampus receives its neocortical input from the entorhinal cortex, which communicates with the perirhinal and parahippocampal cortices, it seems logical that impairing the function of these parahippocampal structures could alter nomal hippocampal function, as well; after all, inactivating the entorhinal cortex effectively dissociates the hippocampus from its neocortical information source.

As described above, the entorhinal cortex is the main gateway through which

neocortical information reaches the hippocampus. Once in the hippocampus, the information is transported via a series of mostly unidirectional, excitatory synapses (Figure 5). Information travels from the entorhinal cortex along the perforant pathway to the dentate gyrus. The granule cells, the principal cells of the dentate gyrus, send their axons, known as mossy fibers, to the pyramidal cells of the CA3 region. In turn, the CA3 region projects via Schaffer collaterals to the pyramidal cells of CA1. Projections from CA1 are slightly more variable: CA1 projects to the subiculum and also sends directly reciprocated projections to the entorhinal cortex. The subiculum projects to the presubiculum, parasubiculum and also to the entorhinal cortex (Amaral & Lavenex, 2007).

The pyramidal cells, the principal cells of the CA1 region of the hippocampus appear especially important in memory functioning. The pyramidal cells are glutamatergic and express receptors that mediate both excitatory and inhibitory transmission. Glutamate, the main excitatory transmitter of the hippocampus, activates three principal types of excitatory receptors: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), kainate and *N*-methyl *D*-aspartic acid receptor (NMDA). AMPA receptors are composed of various combinations of GluR1 - 4 subunits; they are present at excitatory synapses and open in response to a rapid pulse of glutamate. Kainate receptors are also made up of GluR subunits, and activate in response to glutamate binding. NMDA receptors, which open later and stay open longer than AMPA and kainate receptors, facilitate a massive Ca^{2+} influx that leads to the induction of long-term potentiation (LTP) (Bliss, Collingridge, & Morris, 2007). Blocking NMDA receptors (Morris, Anderson, Lynch, & Baudry, 1986) or injecting a calcium chelator

both block LTP and impair learning and memory (Bliss et al., 2007). Similarly, blocking AMPA receptors prevents the initial depolarization necessary to relieve the Mg^{2+} block from NMDA receptors, thereby indirectly blocking the Ca^{2+} influx necessary for LTP.

In addition to excitatory transmission, the dorsal CA1 region also has in place inhibitory mechanisms which prevent over-excitation that could lead to seizures and play an important role in the habituation effects resulting from long-term depression (LTD). These inhibitory mechanisms are in part mediated by the $GABA_A$ receptors, which are located on the cell bodies and proximal dendrites of the pyramidal cells. $GABA_A$ receptors, unlike $GABA_B$ receptors, are ionotropic and heteropentameric (Figure 6). There are at least seven different subunits that are possible members, but most hippocampal $GABA_A$ receptors are composed of two α subunits, two β subunits and either a γ or δ as the fifth subunit. The receptor has two GABA binding sites; when two molecules of GABA (released from inhibitory interneurons within the CA1) bind to the receptor, the chloride (Cl^-) channel opens. The resulting Cl^- influx hyperpolarizes the neuron, preventing the neuron from depolarizing and firing action potentials. Neuronal activity is also affected by a number of modulators, such as benzodiazepines, barbiturates, ethanol and neurosteroids, as well as a number of agonists and antagonists that can act on the $GABA_A$ receptors (Kullmann, 2007). Just as AMPA and NMDA receptor antagonists can prevent LTP-induced learning by blocking excitatory glutamatergic transmission, $GABA_A$ agonists, such as muscimol, can block LTP and learning by increasing inhibition. Intrahippocampal muscimol microinfusion has been reported to impair both spatial memory (McHugh, Niewoehner, Rawlins, & Bannerman, 2008) and conditioned fear memory (McEown & Treit).

1.11. Lesion techniques

It is also possible to impair hippocampal function via such techniques as induction of ischemia (Wood & Phillips, 1991) and ibotenic acid or radio-frequency lesion (Clark et al., 2000).The problem with such techniques is that permanent damage makes it impossible to examine the role of the hippocampus in distinct memory stages, such as encoding, consolidation, retrieval and re-encoding. Also, the long-lasting effects of permanent lesions might result in restructuring of the affected memory processes. Fortunately, understanding the cellular mechanisms behind excitatory hippocampal activity presents other methods of inactivation.

With a strong understanding of the neurons in the dorsal CA1 region, we are equipped to pharmacologically manipulate them in order to take a closer look at the hippocampus' role in learning and memory. This can be accomplished in a variety of ways. One can transiently inactivate the hippocampus by blocking excitatory transmission with intracranial infusion of voltage-gated Na^+ channel blockers, such as lidocaine (Hammond et al., 2004), with NMDA receptor antagonists, such as AP5 (Morris et al., 1986), or with AMPA receptor antagonists, such as LY326325 (Riedel et al., 1999). Alternatively, it is possible to temporarily inactivate the hippocampus by increasing inhibition; this can be accomplished with intracranial infusion of a $GABA_A$ agonist, such as muscimol (McHugh et al., 2008).

Several of the aforementioned techniques have improved our understanding of rodent hippocampal function, but they have also faced criticism for their lack of specificity. For example, as Clark, Zola and Squire (2000) acknowledge, radio-frequency lesions also damage fibers of passage, and the excitotoxic cell death caused by ibotenic

acid might cause extrahippocampal pathology. The induction of ischemia utilized by Wood and Phillips could also affect parahippocampal cortices, as has been argued by Mumby *et al.* (1996). Finally, since voltage-gated Na^+ channels are located on both cell bodies and axons, the deficit found in the Hammond *et al.* (2004) study might be attributable to inactivation of the fibers of passage in addition to the inactivation specific to the dorsal CA1 region of the hippocampus. Blockade of NMDA receptors is also problematic: NMDA antagonists cause sensorimotor impairments and affect neuronal processes not limited to LTP (Bliss et al., 2007). Muscimol, a target-specific drug that can transiently inactivate brain region function without directly affecting fibers of passage, has emerged as an ideal pharmacological tool for examining the role of many brain regions—including the hippocampus.

1.12 Current Study: Purpose & Hypothesis

The current study seeks to address the longstanding debate regarding the role of the hippocampus in non-spatial object recognition memory by examining the impairment caused by bilateral intrahippocampal infusion of muscimol prior to the retrieval phase of the task. Results of the current study confirm the hypothesis that the rodent hippocampus is involved in object recognition memory.

Transient inactivation of the hippocampus via intrahippocampal muscimol microinfusion was first demonstrated to impair spatial learning in the Morris water maze, a hippocampal-dependent task. The same infusion process was then utilized in conjunction with a modified novel object recognition paradigm to elucidate the role of the hippocampus during the retrieval stage of object recognition memory. The dramatic deficits exhibited by mice that received intrahippocampal muscimol infusions compared

to the control-treated mice support the conclusion that increasing the inhibition of the dorsal hippocampal neurons impairs object recognition memory.

PART II: GENERAL MATERIALS AND METHODS

2.1. Subjects

The subjects were male 9 – 16 week old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME). Mice were group housed, 4/polycarbonate cage, in a temperature and humidity-controlled vivarium. Mice were acclimated to the colony room for a total of one week before undergoing surgery or preliminary testing. Cages were maintained on a ventilated rack and mice had *ad libitum* access to food and water for the duration of the experiment. The room was maintained at 22 ± 4 °C and 50 ± 5% humidity and was set on a 12 hr light/dark cycle with the light cycle beginning at 7:00 AM. All behavioral testing was completed during the light phase of the cycle. All animal use procedures were conducted in accordance with the guidelines required by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Florida Atlantic University's Institutional Animal Care and Use Committee approved all procedures before these experiments began.

2.2 Surgery

Mice were surgically implanted with chronic bilateral intracranial guide cannulae directed above the CA1 region of the dorsal hippocampus (dCA1). Prior to beginning surgery, each mouse was deeply anesthetized by isoflurane (Webster Veterinary, Devens, MA), vaporized at a rate of 5% per 1 liter of oxygen using a vaporizer and anesthesia chamber (VetEquip, Pleasanton, CA). Once anesthetized, scalp hair was trimmed and the

mouse was then secured in a stereotaxic apparatus (Model 1900, David Kopf Instruments, Tujunga, CA); isoflurane was reduced to 1% - 2% for the duration of surgery. Sterile lubricating ointment (Pharmaderm, Florham Park, NJ) was applied to the mouse's eyes and scalp skin was washed with Betadine scrub, 70% ethanol and then with Betadine solution. The scalp was cut and retracted and the periosteum was retracted to reveal skull sutures. The pitch of the head was adjusted to level the skull between bregma and lambda, and then burr holes were drilled above the dorsal CA1 region of the hippocampus at 2.0 mm posterior to bregma, ±1.5 mm lateral to the midline and approximately 1.6 mm ventral to the surface of the skull (Franklin & Paxinos, 2007). Two posterior anchor screw holes and one anterior anchor screw hole were drilled through to the dura, but not deeper. After the three 1/8" 000-120 jeweler's screws (Small Parts Inc., Miami Lakes, FL) were in place, sterile 26 gauge bilateral guide cannulae (Plastics One, Roanoake, VA) were lowered into the burr holes directly above the dCA1; dental acrylic (Dentsply International Inc., Milford, DE) was used to affix the cannulae to the anchor screws, thereby permanently holding the cannulae in place. The scalp incision was sutured around the base of the acrylic with tissue glue (VetBond, St. Paul, MN), sterile bilateral dummy cannulae (9.1 mm, Plastics One) were inserted to prevent the guide cannulae from becoming clogged and a sterile aluminum dust cap (Plastics One) was firmly screwed in place. Triple antibiotic ointment was applied to the affected area and each mouse received an IP injection of 0.8 mL of sterile 0.9% saline and a subcutaneous injection of buprenorphine (0.5 mg/kg of body weight). The mice were placed in an empty holding cage on a heating pad until they recovered their righting reflex and mobility, and then they were returned to their home cages. Mice were given

medicated water (3 mL ibuprofen/80 mL water) for 48 hours and were monitored for one week while they recovered from surgery.

2.3 Microinfusions

Immediately prior to beginning the infusion session, 10 mg of muscimol was dissolved in 10 mL of nanopure water. The aCSF was also made fresh on the day of infusion with the following concentrations in distilled H_2O: 147mM NaCl, 2.9mM KCl, 1.6mM $MgCl_2$, 2.2mM dextrose, 1.7mM $CaCl_2$-2 H_2O, 35.9mM $NaHCO_3$. The final solution was adjusted with HCl to a final pH of 7.4 ± 0.2. One mL of each solution was transferred via pipette into a sterile aliquot, labeled with an arbitrary color by a different lab member in order to maintain experimenter blindness and vortexed immediately prior to drawing the solution up into the infusion cannulae.

All mice received bilateral intracranial microinfusions 60 min prior to the testing session. At the time of infusion, the mice were gently restrained, the dust caps and dummy cannulae were removed and microinfusion cannulae were inserted through the guide cannulae to permit microinfusion into the dorsal CA1 subfield of the hippocampus. Microinfusion cannulae were connected to 10 µL Hamilton syringes (Hamilton Company, Reno, NV) that were mounted in a CMA 400 Microinfusion Syringe Pump (CMA Microdialysis, Solna, Sweden). A total volume of 0.5 µL of either artificial cerebrospinal fluid (aCSF) or muscimol (Tocris, Ellisville, MO; 0.5µg of muscimol per side) was administered to each mouse over 3 min at a flow rate of 0.167 µL/min. After the infusion was complete, microinfusion cannulae remained in place for an additional 2 min to allow for diffusion of the perfusate. After the intracranial infusion, dummy cannulae and dust caps were replaced and mice were placed in holding cages (2 per cage

for NOR, 4 per cage for MWM) for 60 min prior to the commencement of behavioral testing.

2.4 Histology

Upon completion of an experiment, all mice from that experiment received intrahippocampal microinfusions of Cresyl violet, a Nissl stain, in order to facilitate the subsequent histological verifications. The microinfusion process was identical to that used for muscimol and aCSF infusions: 0.5 µL per side was infused at a rate of 0.167 µL/min. After the 3 min infusion was complete, microinfusion cannulae remained in place for an additional 2 min to allow for diffusion of the stain. Approximately 60 min later, mice were euthanized by isoflurane overdose (Webster Veterinary). The brains were dissected and placed in 4% paraformaldehyde. Over the following days, the brains were cryoprotected in 20% then 30% sucrose in paraformaldehyde. Once fixed, the brains were sectioned into 50 µm coronal sections on a sliding microtome (Leica Microsystems Inc., Bannockburn, IL) at –19° C with a Physitemp freezing stage, mounted and stained with Neutral Red, a Nissl stain used as a counterstain with the previously infused Cresyl violet. Coverslips were secured in place with histological mounting medium (National Diagnostics, Atlanta, GA) and the slides were left to dry. After they were dried and cleaned, slides were examined with a Nikon Eclipse 55i microscope in order to verify cannula placements and effective infusions, as indicated by a disruption in the pyramidal cell layer of the dorsal CA1 region (Figure 7). Representative images of the microinfusion locations for each mouse were captured using the Nikon Elements software package running on a PC interfaced to the Nikon Eclipse microscope. The data for any mouse that was determined to have inappropriately placed infusion cannulae were

excluded from the analyses, as were data for all mice who either reacted poorly to the intrahippocampal muscimol infusion or were held prior to testing with a mouse that reacted poorly, resulting in the sample sizes indicated in the subsequent sections.

PART III: MORRIS WATER MAZE

3.1 Morris Water Maze Materials

A 62 cm tall cylindrical pool, measuring 1.18 m in diameter, was filled with water and made opaque by the addition of non-toxic white gothic powder paint (Sargent Art, Inc., Hazleton, PA). The water was maintained at a temperature of 23.0 ± 1 °C for the duration of the experiment. The escape platform, a 46 cm tall clear plastic cylinder with a diameter of 7.60 cm, was submerged 1 cm below the surface of the water. (See Figure 8 for a diagram of the pool and its defined zones.) The pool was surrounded by black curtains which were suspended from a circular track, approximately 45 cm outside the edge of the pool. Mouse behavior while in the arena was captured with Ethovision 7.1 (Noldus Information Technology, Leesburg, VA) via a video camera centered above the pool and suspended 1.1 m above the surface of the water; the live feed was displayed on a computer screen approximately 1.4 m from the SE edge of the pool. The curtain was always positioned so as to conceal the computer and the experimenter from the mouse in the pool.

3.2 Morris Water Maze Behavioral Testing (Figure 9)

3.2.1 Habituation & Training

After surgery, the mice were gradually habituated to the testing environment and to being handled. They were transported into the laboratory and weighed twice during the week after surgery. Approximately 7 days after surgery, Morris water maze (MWM)

habituation began.

On Day 1, the curtains were closed around the pool, eliminating any visual cues. The escape platform was placed in the center of the pool and submerged 1 cm below the surface of the water. Mice were transported into the testing room in a polycarbonate cage that contained all four mice from a given home cage. Each mouse received an individual pool habituation session: the mouse was placed on the platform for a total of 60 s. If the mouse jumped off the platform, the experimenter would stop the stopwatch, return the mouse to the platform, restart the stopwatch and hold him on the platform by the tail for the reminder of the session. After the mouse accrued 60 s on the platform, he was placed in a holding cage outside the curtain. After all mice from the home cage were given a habituation session, the cage was transported out of the enclosed testing room into the main laboratory room. The cage was placed on a wire shelving unit and covered with a wire top. On the shelf above the mice, an electrical heater was positioned so as to stream warm air at the mice below. The mice were left to dry and warm up for approximately 20 min before their holding cage was moved to the lab bench. Approximately 2 hours later the process began again, so that each mouse received a total of two habituation sessions on Day 1. After drying from the second habituation session, the mice were returned to their home cages. Once all trials were completed, the home cages were returned to the colony room.

On Days 2 – 5, the curtains were partially retracted and visual cues were suspended from the fabric of the curtains and from the walls visible through the openings in the curtains. Identical to the habituation day, mice were transported into the testing room in a holding cage that contained all mice from a given cage. Mice were trained to

swim to the escape platform, which was located in the SW quadrant of the pool, 27 cm from the pool wall and submerged 1 cm below the surface of the water. Mice were each given four trials per day, one from each of the four starting locations (N, S, E, W). On any given trial, the mouse was carried in the hand of the experimenter from the holding cage to the starting position. The mouse was placed in the water facing the wall of the pool, and the experimenter stepped behind a curtain, out of sight. Once the mouse had been placed in the pool, the Ethovision program automatically began tracking its swim path. When the mouse climbed onto the escape platform, Ethovision automatically ended the trial. The mouse was left on the platform for 30 s, and then the experimenter returned the mouse to the holding cage behind the curtain. If the mouse ended the session by climbing onto the platform but then jumped off, the experimenter returned the mouse to the platform until the full 30 s on the platform had been accrued. If after 60 s of swimming, the mouse did not find the platform, then the experimenter stepped out from behind the curtain and placed his or her finger on the platform to guide the mouse. If the mouse didn't swim to the platform, the experimenter placed him on the platform. Once the mouse was on the platform, the experimenter stepped out of view and began the stopwatch, returning only if the mouse left the platform before 30 s had expired. Once the mouse had been on the platform for 30 s, he was returned to the holding cage. All mice in the cage received their first trials, and then all received their second trials, and so forth, until each mouse had received four training trials. The order of the cardinal directions at which the mice were placed in the pool varied across training days. Once all mice had received the four training trials, the holding cage was carried out of the testing room and placed under the warm air stream for approximately 20 min. Once dry, the mice were

returned to their home cage; when all trials were completed the mice were returned to the colony room.

On Days 3 – 5, in addition to the training procedure, each mouse received mock infusions: the mice were gently restrained and bilateral dummy infusion cannulae were inserted through the guide cannulae and left in place for 5 min. Since all mice from a cage (up to 4) were transported into the testing room together, the mock infusions for MWM testing included four set-ups. Four mice received mock infusions simultaneously, but were each placed in individual holding cages during the mock infusion process. Mice were permitted to move freely within individual uncovered holding cages while the dummy infusion cannulae were in place. The dummy cannulae were connected to empty polyethylene tubing (PE 20, Becton Dickinson and Company, Sparks, MD). These dummy infusion cannulae did not project beyond the implanted tip of the guide cannulae; this way each mouse was habituated to all procedures related to the microinfusion process without affecting the dCA1.

Once the 5 min mock infusion process ended, dummy cannulae and dust caps were replaced and the mice were moved together into one holding cage. After 60 min the mice were transported into the testing room and training began.

After each day of training, each mouse's individual latency to escape (measured as the amount of time, in seconds, it took for the mouse to climb onto the submerged platform) and mean velocity (cm/s) for each trial was calculated and analyzed. Training continued until mice reach asymptote, no longer decreasing latency from day to day. This resulted in four complete days of training (Days 2-5).

3.2.2 Testing

On Day 6, the probe test, mice received microinfusions of either aCSF or muscimol, as described in section 1.3. At the completion of the infusion process, mice were placed in polycarbonate holding cages with the other mice from their home cage. Mice were left in holding cages for 60 min, at which time they were transported into the enclosed testing room. Visual cues and water level and temperature were all identical to the training days; however, the platform had been removed. Each mouse received one 60 s probe, starting at the arbitrarily selected north starting point: the mouse was placed into the water facing the wall of the pool and left to swim for 60s. All trials were recorded by Ethovision. After 60 s the trial automatically ended. The experimenter removed the mouse from the pool and placed him back in the holding cage. When all mice from a given cage had completed the testing session, the holding cage was transported out of the testing room and placed under the heat fan for approximately 20 min. Once dry, the mice were returned to their home cage and, once all trials were completed, the mice were returned to the colony room.

3.2.3 Behavioral Analyses

Learning across training trials and days was assessed as latency to escape, the amount of time it took for a mouse to climb onto the platform and end the trial. Individual swim velocities were averaged for each training day; these data were later used to assess whether aCSF or muscimol induced any locomotion-ability alterations.

Each mouse's performance in the probe was quantified in a number of ways:

- Quadrant dwell times were calculated to determine how much time each mouse spent in the target quadrant and in each of the alternative quadrants during the probe test. For this measure, chance is 15 s.

- Southwest (SW) search ratio was calculated as a more precise indication of each mouse's search accuracy. SW Search Ratio is the frequency of entries into the SW search zone divided by the sum of entries into all four search zones. For this measure, chance is 0.25.
- A measure of exact search accuracy, the RIOS (Real Instances of Success) platform:pool ratio, a new measure developed in the current study, was determined by calculating the time spent in the exact target location, the platform zone, as a function of total time spent in the testing arena. The relative sizes of the arena and the platform predict that the chance ratio of time spent in the platform zone would be 0.004148. Each treatment group's average time spent in the platform zone was compared to chance in order to determine whether search accuracy was significantly different from chance.
- Each mouse's mean distance from the platform center location was calculated and used as a measure of how close the mouse was to the target location throughout the duration of the probe test.
- Finally, each mouse's mean velocity was calculated, averaged by treatment group, and compared to the previous day's mean velocity in order to determine whether aCSF or muscimol affected swim ability during the probe test.

3.2.4 Statistical Analyses

One-way ANOVAs were used to compare test-day search behaviors and swim velocities between three subgroups. Repeated measures ANOVAs were conducted to

analyze escape latency and swim velocity across training, followed by a post hoc multiple comparisons Tukey's Honestly Significant Difference (HSD) test or a two-tailed Student's *t*-test where appropriate. Levene's Test for Equality of Variances was conducted to determine whether variance between groups was equal, then the appropriate independent groups Student's *t*-test (with or without equal variance assumed) was conducted to determine significance between groups. Paired-sample Student's *t*-tests were used to determine significance within groups between the last day of training and the probe test. One-sample Student's *t*-tests were used to determine significance between a group and chance. All statistical analyses were conducted using the PASW v18 (IBM, Somers, NY) software program. Data are presented as mean ± SEM with significance set at $P < 0.05$.

3.3 Morris Water Maze Results

3.3.1 Definition of Groups

The experimental group (n = 8) all received intracranial bilateral dorsal CA1 microinfusions of muscimol, and will heretofore be referred to as the muscimol group. The control group (n = 7) consisted of three subsets: 3 mice received intracranial bilateral dorsal CA1 microinfusions of aCSF, 1 mouse received a unilateral dorsal CA1 microinfusion of aCSF because of a complication during the infusion process, and 3 mice received mock infusions. A one-way ANOVA was conducted in order to compare these three subsets on two different measures. There was no significant difference in SW Search Ratio between any of the three subsets (Figure 10; $F_{2, 6} = 0.136$, $P = 0.877$), nor was there any significant difference in test-day swim velocities between the three subsets (Figure 11; $F_{2, 6} = 5.801$, $P = .066$). Based on these results, it was concluded that behavior

of these three subsets was not significantly different in any way; therefore, the aCSF, unilateral aCSF and mock infusion mice were combined into one group, referred to as the control group. No further distinction will be made between the different subsets.

3.3.2 Acquisition

Over the course of training mice became progressively more efficient at locating the platform, as indicted by decreased latency to escape times (Figure 12). A two-way repeated measures ANOVA revealed that there was a statistically significant difference in latency to escape between days ($F_{3,39} = 17.863$, $P < 0.001$); however, there was not a significant difference between future treatment groups ($F_{1,13} = 0.002$, $P = 0.962$), nor was there a significant difference in the day x future group interaction ($F_{3,39} = 1.256$, $P = 0.303$). These results indicated that prior to test-day infusions, the future groups were indistinguishable in terms of acquisition of the MWM task.

An analysis of total distance to point (TDP), a measure of each animal's cumulative distance from the platform center throughout the duration of the trial, compared across training verifies this conclusion (Figure 13). A two-way repeated measures ANOVA revealed that although there was a significant difference in TDP across training days ($F_{3,39} = 28.158$, $P < 0.001$), there was not a significant difference between future treatment groups ($F_{1,13} = 0.057$, $P = 0.815$), nor was there a significant difference in the day x future group interaction ($F_{3,39} = 1.100$, $P = 0.361$).

Mean swim velocity was analyzed to further compare groups on the last day of training (Figure 14). An independent samples Student's *t*-test demonstrates that the future groups had similar swim velocities on the last day of training ($t_{13} = -0.368$, $P = 0.719$).

3.3.3 Probe Test

Each group's probe test quadrant preference (Figure 15) was analyzed with a one-way ANOVA and post hoc Tukey's HSD test. Results indicated that quadrant dwell times were significantly different between groups ($F_{3, 27} = 8.590$, $P < 0.001$). Post hoc Tukey's HSD revealed that the time control mice spent in the target SW quadrant was significantly greater than the amount of time spent in the NW quadrant, $P = 0.008$, and significantly greater than the amount of time spent in the NE quadrant, $P < 0.001$. The control mice spent more time in the SW quadrant than the SE quadrant (mean difference = 6.29 s), but this difference was not significant, $P = 0.092$. On the other hand, the same tests revealed that the muscimol group spent significantly *less* time in the target SW quadrant than in the NW quadrant, $P = 0.037$, or in the NE quadrant, $P = 0.001$. This group spent significantly more time in the NW quadrant than in the SE quadrant, $P = 0.013$, and significantly more time in the NE quadrant than in the SE quadrant, $P < 0.001$.

Independent group Student's *t*- tests were conducted to further analyze quadrant dwell times between groups. These analyses revealed that there were significant differences between the amount of time the muscimol versus the control groups spent in the target SW quadrant ($t_{13} = 3.986$, $P = 0.002$), the NE quadrant ($t_{13} = -4.496$, $P = 0.001$) and the SE quadrant ($t_{13} = 2.750$, $P = 0.017$). The groups did not spend a significantly different amount of time from each other in the NW quadrant, although this difference did approach significance ($t_{13} = -2.093$, $P = .057$). These results revealed that the control group spent significantly more time in the target SW quadrant, but the muscimol group did not, indicating that the control group's spatial navigation memory was intact but the muscimol group's spatial navigation memory was impaired.

A more specific measure, SW search zone ratio, further supported this conclusion

(Figure 16). SW search zone ratio is the time spent in the SW search zone divided by the total time spent in any of the 4 search zones. An independent samples Student's *t*-test revealed that the control group spent significantly more time in the SW search zone than did the muscimol group (t_{13} = 4.973, P < 0.001). Additionally, a one-sample Student's *t*-test was conducted for each group to compare time spent in the SW search zone to chance (0.25; Figure 16): the average time the control mice spent in the SW search zone was significantly greater than would be predicted by chance (t_6 = 4.619, P = 0.004), while the average time the muscimol mice spent in the same zone was less than chance, but not quite significantly so (t_7 = -2.204, P = 0.063).

The RIOS platform:pool ratio was assessed by calculating the time spent in the exact target location, the platform zone, as a function of total time spent in the testing arena (Figure 17). One-sample Student's *t*-tests revealed that the control group spent significantly more time (0.6345861 s) in the platform zone than would be predicted by chance (chance = 0.004148 s; t_6 = 5.952, P = 0.001), but the muscimol group (0.1376605 s) did not (t_7 = 1.691, P = 0.135). The time spent in the platform zone was also significantly different between groups (t_{13}= 3.758, P = 0.002).

It is important to note that the differences between groups were not a result of impaired locomotion or motivation. This was demonstrated by a paired-samples Student's *t*-test comparing each group's velocity on the last day of training with its velocity on the probe test (figure 3.11). The muscimol group did not demonstrate any significant difference in velocity from one day to the next (t_7 = -1.025, P = 0.340). Unexpectedly, the control group's velocity increased significantly on the probe test (t_6 = -9.053, P < 0.001). The muscimol group's velocity on the probe test was significantly

slower than that of the control group ($t_{13} = 2.297$, $P = 0.039$). However, in light of the aforementioned within-subjects Student's *t*-tests, it is clear that this difference is not a result of a decrease in velocity of the muscimol group; rather, it is a result of an increase in velocity of the control group. It is unclear why this increase occurred, but it does not diminish from the finding that the swim speed of muscimol treated mice was unimpaired on the test day as compared to their swim speed on the previous training day.

Together, these results indicate that intracranial bilateral dorsal CA1 microinfusions of muscimol effectively impair performance on the Morris water maze, a well-established hippocampal-dependent task (Morris et al., 1982). It can be inferred that the muscimol microinfusions effectively impaired hippocampal function without affecting locomotion.

3.4 Morris Water Maze Discussion

The Morris Water Maze (MWM), a well established hippocampal-dependent task (Morris et al., 1982), was utilized in order to confirm that intrahippocampal microinfusion of muscimol effectively impairs hippocampal function. The muscimol group's significant impairment on the probe test replicated Riedel *et al.*'s finding (1999) that, in addition to a role in other memory processes, the hippocampus is necessary specifically for the retrieval of spatial memory. Furthermore, the muscimol group's impairment indicated that the current infusion process successfully deactivated the hippocampus 60 min after infusion.

Although the current data present a convincing argument that spatial navigation was impaired, they fall short of demonstrating the severity of the impairment that the intrahippocampal muscimol group exhibited on the probe test. Not only did these mice

fail to successfully navigate to the target zone and to show any persistence in searching there, but they also failed to demonstrate effective search behavior in any other quadrant. If the rodent hippocampus is necessary only for spatial navigation memory, then the muscimol group would be expected to demonstrate a memory of the task and might even exhibit effective search strategies in the wrong region; however, this was not the case. As illustrated by the swim path images (Figure 18), the mice who had received intrahippocampal infusions of muscimol generally behaved similarly to mice that are placed into the water maze for the first time: they swam along the edge of the pool and pawed at the wall in an effort to climb out of the pool. Mice tend to exhibit this sort of behavior on their first day of training, but quickly learn that it is not an effective escape strategy. As exhibited by their decreased escape latencies across training days, the future intrahippocampal muscimol group learned that this strategy was not effective, but based on their test-day performance, the retrieval of this memory was blocked along with the memory of any spatial representations. This conclusion, that the muscimol infusion impaired the retrieval of the memory of the task in addition to the memory of the spatial representation, seems to indicate that the hippocampus is, indeed, necessary for more than just spatial navigation memory.

PART IV: NOVEL OBJECT RECOGNITION

4.1 Novel Object Recognition Materials

The two 38 x 38 x 64 cm high acrylonitrile butadiene styrene (ABS) arenas were located in the testing room, next door to the main laboratory. Mouse behavior while in the arena was captured with Ethovision XT (Noldus Information Technology, Leesburg, VA) via a video camera suspended 150 cm above the arena floor directly above the border walls separating the two arenas; the live feed was displayed on a computer screen approximately 4.7 m from the testing arena, where the video file was automatically saved. (See Figure 19 for a diagram of the NOR arena and objects.)

4.1.1 Subject Clarification

Out of the 17 total mice used in the Novel Object Recognition (NOR) testing, 8 of them underwent NOR testing 21 days prior to this experiment: they were used in the same arena but did not have prior exposure to the "novel" object used in this round of experiments. The experimental procedure followed with this cohort of mice in the current study was identical to that followed with the naïve mice; however, it should be noted that this cohort was not naïve to the infusion process, the arena or the familiar object used in the current experiment. The non-naïve mice had received artificial cerebrospinal fluid (aCSF) or muscimol microinfusions during their first round of testing; the microinfusions administered to these mice in the current experiment were counterbalanced so that half of the mice received the same infusion as before (aCSF-aCSF or muscimol-muscimol) and

the other half received a different infusion than before (aCSF-muscimol or muscimol-aCSF). All mice that received muscimol behaved similarly regardless of whether they had received muscimol or aCSF 21 days prior and all mice that received aCSF in the current experiment behaved similarly regardless of whether they had received aCSF or muscimol 21 days prior; in other words, the results obtained from the mice that received identical treatments in both rounds of testing did not vary from the mice that received different infusions. Furthermore, the results of the aCSF and muscimol non-naïve groups did not vary significantly from the aCSF and muscimol naïve groups, respectively, so the groups were analyzed together. Aside from the presentation of statistical evidence of the groups' similarities in the results section and an interpretation of the implications of the similarity in the discussion section, no further distinction between the groups will be made.

4.1.2 Preliminary Object Preference and Arena Testing

Prior to beginning NOR testing, it was important to establish that the object pair to be used generated appropriate exploration. Specifically, mice should demonstrate equal preference when both objects are novel, and should demonstrate significant novel object preference when either one of the objects is more novel than the other. Furthermore, it was necessary to establish that the arena itself did not generate any location-specific preference. These pre-requirements were established in preliminary studies with a cohort of non-cannulated, naïve mice (N = 24). The mice utilized in the preliminary study were not used again in any part of the current study.

The preliminary testing was a 5 day process. On Day 1, the mice were habituated to the holding room, the polycarbonate holding cages and to being handled; their tails

were marked with a blue Sharpee marker to permit identification. On Days 2 – 4, the mice were habituated to one of the two empty arenas (n = 8), an arena with two identical "spring" objects (n = 8), or an arena with two identical "feet" objects (n = 8). In the "spring" and "feet" conditions, either two metallic anchor feet affixed to individual Plexiglas bases or two springs affixed to individual Plexiglas bases were placed in the arena, approximately 3 cm from the NW and NE corners (Figure 19). Each habituation session lasted for 10 min, during which time the mouse was tracked with the Ethovision XT system. The arenas and objects were cleaned with a 10% ethanol solution between each trial in order to diminish the possibility that future mouse performance was influenced by odor cues left by the previous mouse. After all habituation sessions were completed for a given day, the mice were returned to the colony room.

On Day 5, each mouse was given a 5 min test session with two objects. The mice that had been habituated to an empty arena were exposed to one of each object; therefore, the objects were equally novel. The mice that had been habituated with identical objects (springs or feet) were exposed to one of the identical objects (spring or foot) and one novel object (foot or spring). The respective locations of the foot and spring were counterbalanced across all trials.

After the test sessions were complete, a researcher, blind to habituation condition, manually coded each mouse's exploration time at each object using computer display XNote Stopwatches (dnSoft Research Group, www.dnsoft.swrus.com). A mouse was considered to be exploring the object if it was facing the object and its nose was within 2.0 cm of the object. Time spent grooming beside the object or using the object merely to climb higher while facing away from the object was not considered exploration time.

Successful retention of object memory was inferred if the mouse exhibited a preference for exploring the novel object over the familiar one during the test session; this was quantified by calculating the Novel Object Preference Ratio (NOPR; time spent exploring novel object divided by total time spent exploring both objects).

4.2 Novel Object Recognition Behavioral Testing (Figure 20)

4.2.1 Habituation/Sample

Mice used in the current experiment were gradually habituated to the testing environment and to being handled. They were transported into the laboratory and weighed twice during the week after surgery. Approximately 7 days after surgery, behavioral testing began. The first five days of testing served to gradually habituate the mice to the testing environment, the testing arena and to the microinfusion process.

On Day 1, mice were transported into the laboratory and left in their home cages on the lab bench for one hour. After the one hour room habituation, mice were weighed and tail markings were re-colored using a blue Sharpie marker. Mice were then transferred into polycarbonate holding cages with the one other mouse with whom they would be in holding cages after infusions and before testing. After 10 min in the holding cages, mice were transferred back into their home cages and returned to the colony room.

Days 2, 3 and 4 served to acclimate the mice to the intracranial microinfusion procedure, the testing arena and the familiar objects. The mock infusions were identical to those described in section 5.1.1, except that since only two mice were transported into the testing room together, the mock infusions for NOR testing included only two set-ups. Two mice received mock infusions simultaneously, but were each placed in individual holding cages during the mock infusion process. Once the 5 min mock infusion process

ended, dummy cannulae and dust caps were replaced and the mice were moved together into one polycarbonate holding cage.

After 60 min, the mice were transported via the holding cages into the testing room where they were placed individually in one of two NOR arenas for 10 min. Each arena contained two identical objects (either two feet or two springs) one in the NW corner and one in the NE corner (Figure 19). After 10 min, mice were returned to polycarbonate holding cages, transported back into the infusion room and returned to their home cages. The arenas and objects were cleaned with a 10% ethanol solution between each trial in order to diminish the possibility that future mouse performance was influenced by odor cues left by the previous mouse. After all habituation sessions were completed for a given day, the mice were returned to the colony room.

4.2.2 Testing

On the fifth day, the test session, mice received bilateral dCA1 infusions of 0.5 µL/side of either artificial cerebrospinal fluid (aCSF) (n=10) or muscimol (1µg/µL, resulting in 0.5 µg of muscimol per side) (n= 7) and were then placed in pairs into holding cages. After 60 min, the mice were transported into the testing room and placed into the individual testing arenas in which they had been habituated. The testing arena was identical to the habituation/sample session arenas except that the one of the objects in each arena had been replaced with a novel object. The novel and familiar objects, as well as the location of the object that was replaced with the novel object were counterbalanced across mice. Each mouse was allowed to independently explore the objects and arena for exactly 5 min before the test session ended; at that time, the mouse was returned to the holding cage, transported back into the infusion room and returned to his home cage.

Again, the arena and objects were cleaned with a 10% ethanol solution between trials.

4.2.3 Behavioral Analyses

Total sample object exploration was calculated by summing the amount of time each mouse spent exploring either of the familiar objects during the three sample/habituation days. This measure was used to rank mice based on individual motivation to explore; mice were then evenly distributed across control and experimental groups, resulting in groups with equal average motivation to explore. Additionally, cumulative distance traveled (CDT) and velocity while moving were both recorded by Ethovision and averaged over the three sample/habituation sessions in order to establish baselines for each mouse. Mean CDT and velocity during locomotion were then calculated during the test session (after mice received microinfusions) and compared to each mouse's individual baseline in order to assess whether aCSF or muscimol altered mobility or exploratory behavior. These measures also enabled an analysis of how behavior changed across training days. After the test sessions were completed, a researcher, blind to treatment, manually coded each mouse's exploration time at each object exactly as described in Section 4.1.2.

4.2.4 Statistical Analyses

A one-way ANOVA was conducted in preliminary studies to compare novel object preference across three different groups. Repeated measures ANOVAs were conducted to analyze mean velocity while moving, cumulative distance traveled, and object exploration across training and testing days for both groups. Repeated-measures ANOVAs were followed by post hoc multiple comparisons Tukey's HSD test or two-tailed Student's *t*-test where appropriate. Levene's Test for Equality of Variances was

conducted to determine whether variance between groups was equal, then the appropriate Student's *t*-test (with or without equal variance assumed) were conducted. Independent groups Student's *t*-tests were conducted to determine significance between groups, paired-sample Student's t-tests were used to determine significance within groups between the last or first day of training and the probe test, and one-sample Student's *t*-tests were used to determine significance between a group and chance. All statistical analyses were conducted using the PASW v18 (IBM, Somers, NY) software program. Data are presented as mean ± SEM with significance set at $P < 0.05$.

4.3 Novel Object Recognition Results

4.3.1 Preliminary Studies

Based on the amount of time the mice spent in the different zones of the arenas, as averaged over all 3 habituation sessions, it was clear that the arenas did not generate any intrinsic location preference (Figure 21). Paired sample Student's *t*-test's indicated that the difference in the average amount of time spent in either object location zone (A or B) was insignificant in Arena 1 ($t_{34} = 0.643$, $P = 0.524$) and insignificant in Arena 2 ($t_{34} = 0.058$, $P = 0.954$). These results indicated that within the two arenas, neither object placement zone was preferential over the other.

In the preliminary object-preference studies, it was determined that the object pairs to be used in testing generated appropriate exploration depending on their novelty (Figure 22). When the objects were equally novel, they generated equal exploration; the novel object preference ratio (arbitrarily calculated for the foot as novel, even though objects were equally novel) was determined to be 0.5096, which is not statistically different from chance ($t_7 = 0.410$, $P = 0.694$). However, when the mice had 30 minutes of total

exploration time (10 min per day x 3 days) 24 h prior to the test session with either of the objects, they subsequently spent significantly more time exploring the novel object than would be expected by chance. This novel object preference was observed when the foot was novel ($t_7 = 9.514$, $P < 0.001$) and when the spring was novel ($t_7 = 8.760$, $P < 0.001$). Furthermore, a one-way ANOVA revealed that novel object preference was significantly different across novelty conditions ($F_{2, 23} = 23.239$, $P < 0.001$). A subsequent Tukey's HSD test revealed that novel object preference exhibited by mice without any prior object exploration was significantly different than novel object preference exhibited by mice with prior foot exploration, $P < 0.001$, and those with prior spring exploration, $P < 0.001$. Tukey's HSD revealed that the novel object preferences exhibited by the groups with prior exposure to either the foot or the spring were not significantly different from each other, $P = 0.980$. These results indicated that the objects to be used in testing elicited equal exploration when they were equally novel and that both objects elicited significantly more exploration when they were novel and presented with the familiar other.

4.3.2 Habituation/Sample Sessions

Cumulative distance traveled (CDT) was analyzed across the three sample days to establish whether there were any significant differences between future groups (Figure 23). An independent samples Student's *t*-test revealed that the two future groups traveled similar cumulative distances on the first day of training ($t_{15} = -1.304$, $P = 0.212$). A two-way repeated ANOVA further revealed that there was not a significant difference in CDT across the course of the experiment between the future groups ($F_{1, 15} = 1.878$, $P = .191$), nor was there a significant interaction between the day and the group ($F_{1, 15} = .030$, P =

.865); however, there was a significant difference between days ($F_{1, 15} = 84.829$, $P < 0.001$). CDT was significantly greater on Day 1 than on Day 2, $P < 0.001$ or Day 3, $P < 0.001$. CDT was also greater on Day 2 than on Day 3, but this difference was only marginally significant, $P = 0.051$. This significant decrease in CDT over the 3 sample sessions is evidence of habituation to the environment over the course of training.

Total object exploration across training was also averaged by future group in order to further establish group similarities (Figure 24). A two-way future group x day ANOVA revealed that there was not a significant difference between the future groups' object exploration ($F_{1, 15} = 0.377$, $P = 0.548$), nor were there any significant differences across days ($F_{1, 15} = 2.955$, $P = 0.106$) or in the group x day interaction ($F_{1, 15} = 0.267$, $P = 0.613$). These results indicated that prior to test day microinfusions the groups were, for all intents and purposes, identical.

4.3.3 Test Session: Principle Results

The current hypothesis, that the function of the rodent hippocampus is necessary for object recognition memory, was confirmed by the results of the NOR test session. Preference for the novel object was assessed as novel object preference ratio (NOPR). An independent subjects two-tailed Student's *t*-test demonstrated that the NOPR of the intra-hippocampal aCSF group was significantly higher than that of the intra-hippocampal muscimol group (Figure 25, $t_{15} = 2.640$, $P = 0.019$). One-sample *t*-tests revealed that the mean NOPR of the aCSF group was significantly higher than chance ($t_9 = 5.745$, $P < 0.001$), but that of the muscimol group was not ($t_6 = 1.532$, $P = 0.176$). The significant NOPR of the mice that received aCSF microinfusion indicated that the 30 min of cumulative sample time with the familiar objects were sufficient to elicit a strong

preference for the novel object when it was presented during the test session. Since the intra-hippocampal muscimol group did not show demonstrate a novel object preference any different from chance, it can be concluded that the temporary hippocampal inactivation prevented the mice that received muscimol micrinfusions from retrieving the memory of the familiar objects. Other measures, such as discrimination ratio (the difference in exploration time between the familiar and novel object divided by the total object exploration) produced results with the same significance as those analyzing the NOPR; therefore, these results are not separately described. The NOPR results offer strong support for the hypothesis that the hippocampus is necessary for the retrieval of object recognition memory.

4.3.4 Test Session: Additional Results

In order to establish that intra-hippocampal muscimol did not impair locomotion, a two-tailed independent samples Student's *t*-test comparing the mean velocity of the groups on test day was conducted (Figure 26). The results indicate that the average test-day velocity of the muscimol mice was significantly higher than the average test-day velocity of the aCSF mice (t_{15} = -3.918, P = 0.001). These results were at first unexpected, but a closer analysis of velocity change as a function of day provided an explanation.

A two-way group x day repeated measures ANOVA (Figure 27) revealed that there was no significant difference of velocity between groups ($F_{1, 15}$ = 3.757, P = 0.072); however, within subjects there was a significant effect of day ($F_{3, 45}$ = 17.293, P < 0.001) and a significant day-group interaction ($F_{3, 45}$ = 4.799, P = 0.006). A one-way ANOVA comparing the velocities of the control group across the duration of the experiment

revealed that there was a significant effect of day ($F_{3, 39} = 4.053$, $P = 0.014$). Tukey's HSD post hoc test indicated that the control group's mean velocity gradually decreased across the three sample days; the difference between Day 1 and Day 3 was significant, $P = 0.012$, but the differences between Day 1 and Day 2, $P = 0.085$, and Day 2 and Day 3, $P = 0.848$, were not significant. The control group's mean velocity showed a slight increase on the test day. This was not a significant change from the day before, $P = 0.884$, but it was still significantly less than velocity on Day 1, $P = 0.085$. These results, combined with the CDT training data (Figure 28), paint a picture of a gradual habituation to the testing arena.

The future intra-hippocampal muscimol group exhibited a similar habituation during the sample days. A one-way ANOVA comparing the muscimol group's mean velocity across the duration of the experiment revealed a significant effect of day ($F_{3, 27} = 8.279$, $P = 0.001$). Tukey's HSD post hoc test indicated that this group also gradually habituated to the testing arena, as demonstrated by a decrease in velocity across the three sample days. The mice moved significantly slower on Day 2 than on Day 1, $P = 0.030$ and significantly slower on Day 3 than on Day 1, $P = 0.002$; however, the decrease in speed from Day 2 to Day 3 was not significant, $P = 0.648$. Unlike the intra-hippocampal aCSF mice, the velocity of the muscimol mice significantly increased from Day 3 to the test day, $P = 0.004$. Also unlike the aCSF group, the average test-day velocity of the muscimol group was *not* significantly different from Day 1 (Figure 27; $P = 0.989$). These results indicate that while all mice gradually habituated to the arena during the three sample days, this habituation carried over into the test day only among the aCSF mice. The intra-hippocampal muscimol group resorted back to behavior similar to that of the

first day, indicating that not only were they unable to retrieve the object recognition memory, but they were also unable to retrieve the memory of the arena.

Similarly, an independent samples Student's *t*-test comparing the intra-hippocampal muscimol and aCSF groups on the day of the test revealed that the aCSF mice traveled significantly less cumulative distance (CDT) than did the muscimol mice on test day (Figure 29; $t_{15} = -3.926$, P = 0.001). A two-way day x group repeated measures ANOVA revealed that there was a significant difference across days ($F_{3, 45} = 68.408$, $P < 0.001$), but not a significant difference in the day x group interaction ($F_{3, 45} = 2.466$, P = 0.074). Since it was already determined that the groups were significantly different on the test day, further analyses were conducted in order to understand how the groups could be significantly different from each other on test day when a day x group interaction was insignificant. Independent samples Student's *t*- test revealed that the intrahippocampal muscimol group's CDT was significantly lower on test day than it was on the first sample day ($t_6 = 5.263$, P = 0.002); however, this is not an indication that muscimol caused impaired locomotion because the intrahippocampal aCSF group's CDT was also significantly lower on test day than it was on the first sample day ($t_9 = 8.644$, $P < 0.001$). The findings that the future groups' CDT values on the first sample day were insignificantly different from each other ($t_{15} = -1.304$, P = 0.212), that both groups' CDT values significantly decreased from the first sample day to the test day but that the intrahippocampal muscimol group's CDT was significantly greater on test day than was the intrahippocampal aCSF group's CDT on test day could be interpreted to indicate that although habituation to the testing arena over the course of the experiment decreased the CDT among both groups, the muscimol microinfusions erased this habituation enough

among the muscimol-treated mice that their CDT was significantly greater than that of the aCSF-treated mice on the test day (Figure 29). These results further supported the conclusions that locomotion was not impaired as a result of muscimol infusion and that the intrahippocampal muscimol mice did not exhibit evidence of habituation to the environment as did the intrahippocampal aCSF mice.

A two-way group x day ANOVA was conducted to compare the treatment groups' object exploration across the experiment (Figure 30). There was not a significant difference in the day x group interaction ($F_{3, 15} = 0.715$, $P = 0.548$), but there was a significant difference of object exploration within groups across days ($F_{3, 15} = 10.533$, $P < 0.001$). Bonferroni pairwise comparisons revealed that the object exploration was significantly different between Day 4 and every other day (Day 4 by: Day 1, $P < 0.001$, Day 2, $P = 0.005$, Day 3, $P = 0.038$). However, this increase in object exploration on the test day was observed in both groups. Independent samples Student's *t*-tests indicated that the two groups spent similar amounts of time exploring the objects each day of the experiment. There was no significant difference between groups' object exploration time on Day 1 ($t_{15} = 0.043$, $P = 0.966$), Day 2 ($t_{15} = 0.950$, $P = 0.357$), Day 3 ($t_{15} = 0.641$, $P = 0.531$) or the test day ($t_{15} = -0.561$, $P = 0.583$).

These results indicated that the intra-hippocampal muscimol group's locomotion was not impaired by the muscimol infusion; in fact, their average velocity on the test day was significantly faster than the aCSF mice, as has been explained. Furthermore, the intra-hippocampal muscimol group did not demonstrate any decrease in motivation to explore: their test-day object exploration was similar to that of the aCSF group. The muscimol group's equal preference for the familiar and novel objects, as compared to the aCSF

group's significant preference for the novel object, indicated that the deactivation of hippocampal function impaired object recognition memory retrieval.

4.4 Novel Object Recognition Discussion

Mice that received intrahippocampal microinfusion of muscimol prior to the test session did not demonstrate a novel object preference (Figure 25), supporting the theory that the hippocampus plays an essential role in object recognition memory. Certainly, this conclusion rests on the assumption that the hippocampus was singly deactivated by the current infusion process; in addition to the histological verifications implemented in this study, future studies are necessary to confirm hippocampus-specific inactivation. This is further discussed in the General Discussion. For the sake of this discussion, it will be assumed that the muscimol-induced inactivation was limited to the hippocampus.

In addition to the NOPR disparity between groups, the muscimol group's increased velocity on test day (Figure 26) further supports the idea that the muscimol infusion resulted in an impairment of memory for both the familiar object *and* the arena. As the mice became habituated to the testing arena over the course of three days, their velocities gradually decreased. The velocity of the control group showed a slight, insignificant increase on the test day, likely as a result of the novel object. The velocity of the intrahippocampal muscimol group, on the other hand, showed a significant increase (Figure 27); it was similar to the velocity on the first day, supportive of the theory that the muscimol mice had no recollection of the testing arena (or of the objects within it). These results support the hypothesis that the rodent hippocampus plays an essential role in non-spatial object recognition memory.

PART V: GENERAL DISCUSSION

Hippocampal inactivation during the test sessions impaired the performance of C57BL/6J mice in both MWM and NOR experiments. These results indicate that the rodent hippocampus is a necessary structure in spatial memory and in non-spatial object recognition memory. These findings are interesting to consider in light of previous NOR studies which, as summarized in Figure 1, vary greatly. Many of the studies indicate that a long delay combined with an inconsistency in the functionality of the hippocampus during an experiment results in impaired novel object recognition (Clark et al., 2000; de Lima et al., 2006; Hammond et al., 2004); studies that induce permanent lesions pre-sample and don't report any impairment (Ainge et al., 2006; Gaskin et al., 2003; Mumby et al., 2005) could also be reconciled with this conclusion. Lesion size is also occasionally presented as a determining factor (Broadbent et al., 2004). The following sections examine these issues more closely and present finding from the current study as a resolution to some of these debates.

5.1 Memory Processes & State Dependency

The current experiments both utilized pre-test infusions, effectively deactivating the hippocampus during the retrieval stage of the MWM and NOR experiments. Future studies could exploit the transience of hippocampal impairment induced by muscimol infusion to examine the effect of pre-training/pre-sample infusions or post-training/post-sample infusions, which would impair hippocampal function during encoding or

consolidation, respectively. In a similar design, Riedel *et al*. (1999) induced reversible hippocampal inactivation by infusing LY326325, a selective AMPA/kainate receptor antagonist, at different MWM stages. They found that chronic hippocampal inactivation prior to all training sessions prevented acquisition of the water maze task and acute inactivation prior to the retrieval stage impaired performance. In addition, they found that hippocampal inactivation at the appropriate times was also effective in interrupting both trace consolidation and long-term memory storage (Riedel et al., 1999). The results of the current MWM study replicate Riedel *et al.*'s findings relevant to retrieval processes. The results of the current NOR study indicate that the hippocampus is also necessary during retrieval of object recognition memory; whether the hippocampus is also necessary during the other stages of object recognition memory remains to be seen. Future studies could implement a similar method of interrupting hippocampal function during distinct stages in order to address the role of the rodent hippocampus specifically during encoding, consolidation and storage of object recognition memory.

The argument has been made that the hippocampus is involved in object recognition memory, but that extrahippocampal structures are sufficient to support such memory in the absence of hippocampal function (Mumby et al., 2005). Mumby *et al.* (2005) found that object recognition memory was impaired when NMDA lesions were induced after the encoding of the object memory, but not when they were induced prior to the encoding stage. That is, they concluded that if an object recognition memory is encoded in the presence of a functional hippocampus, then it can only be retrieved in the presence of the hippocampus. Furthermore, they theorized that if object recognition memory is encoded in the absence of the hippocampus then it can, likewise, be retrieved

in the absence of the hippocampus, indicating that extrahippocampal structures are capable of compensating for an incapacitated hippocampus in object recognition memory.

It is also possible that the successful object recognition memory requires the functionality of the hippocampus to be consistent—whether functional or nonfunctional—across all memory processes (encoding, consolidation and retrieval). After all, in Mumby *et al*.'s study (2005), the lesioned rats that demonstrated normal novel object preference underwent permanent lesions prior to the encoding stage: their hippocampi were compromised during all memory stages. These findings, though limited, seem to indicate that object recognition might be state dependent, requiring the hippocampus to be in the same functional state across memory processes.

Since the Mumby *et al.* (2005) study used a permanent lesion technique, they were unable to examine whether the phenomenon actually was state-dependent; they argue that object memory encoding can be sustained by extrahippocampal structures if necessary, but do not address the issue of whether bringing the hippocampus back on board during the retrieval stage would prevent the effective retrieval of such extrahippocampal-encoded memories. Temporary lesion techniques make it possible to examine the effects of memory stage specific inactivation, enabling a more comprehensive assessment of whether state dependency is a relevant issue in object memory processes.

Although the reversible inactivation methods of the current study permit greater flexibility in hippocampal inactivation, they do not put Mumby's argument to rest; as in Mumby's experiment, object recognition memory in the current study was encoded in the

presence of a functional hippocampus which was then absent during the retrieval stage. Utilizing the current temporary inactivation methods, it would be possible to inactivate the hippocampus during the encoding stage and then observe if the memory can be successfully retrieved 24 h later. According to Mumby's theory, extrahippocampal structures should be sufficient to support the memory encoding, so the mice should perform similarly to controls on a subsequent retrieval stage. However, the Riedel *et al.* (1999) MWM study supports the hypothesis that interrupting hippocampal function during any memory process – encoding, consolidation, storage or retrieval—would impair performance on a spatial navigation task. It will be interesting to elucidate whether the same is true in a non-spatial task by implementing such process-specific inactivation in the NOR task.

5.2 Lesion Issues and Alternatives

The use of muscimol, a $GABA_A$ agonist improves on lidocaine-induced temporary inactivation because, unlike voltage-gated Na^+ channels, $GABA_A$ channels are primarily located on the cell bodies and proximal dendrites of neurons. This means that muscimol, unlike lidocaine, can specifically affect the targeted region without affecting fibers of passage.

While the current lesion technique has many benefits over previously utilized techniques, it is not void of problems. One major issue with the current technique, as with all lesion studies, is the problem of attributing a function to an absent or inactivated structure. Inactivating all structures except for the hippocampus, then examining whether or not the object recognition memory is supported would be a much less debatable technique; of course, if all other structures were inactivated, the animal would not be able

to function, so this method is impossible.

Electrophysiological studies provide an alternative way of examining hippocampal function without requiring its inactivation. Wood *et al*. (1999) recorded 127 CA1 neurons during a modified odor-association DNMS task and found that less than 10% of these neurons exhibited exclusively spatial firing correlates, while 40% of them exhibited exclusively non-spatial firing correlates. Although it remains debatable whether or not the task was a hippocampal dependent task, it is clear that the hippocampus played at least a participatory role in non-spatial elements of the task. These findings, in conjunction with hippocampal lesion studies, paint a picture of the hippocampus as a structure that is, at the very least, involved in non-spatial memory tasks.

5.3 NOR spatiality

This raises another potential criticism of the current study: is the novel object recognition task truly non-spatial? There is reason to believe that it is, indeed, non-spatial: unlike the MWM, NOR does not seem to require any spatial navigation for the animal to perform successfully. On the other hand, it could be argued that the objects in the arena are analogous to the extra-maze cues displayed around the Morris water maze. In this case, it seems that changing the objects on the NOR test day changes the spatial environment with which the animal had become familiar. This line of reasoning makes it seem that the NOR task, though not a spatial task, does possess spatial elements.

However, this argument circles back into an argument for the task's non-spatiality. If visual cues were changed on the probe day of a MWM task, one would not expect animals to perform the task effectively; this impairment would be a direct result of altering the spatial environment. Changing the visual cues on a spatial task would impair

performance because it would prevent retrieval and implementation of the spatial representation. However, the arguably analogous changing of the NOR objects would not impair performance in any way; in fact, it is only through this introduction of a novel object that a novel object preference can be exhibited. If the introduction of a novel object completely altered the spatial environment, then the familiar object would, likewise, be novel in that it is now present in a 'novel' environment. If this was the case, then control mice would be expected to explore both objects similarly, rather than to exhibit novel object preference. Moreover, if changing the object was equivalent to changing the environment, then control mice, like the intrahippocampal muscimol mice, would exhibit behavior similar to their first day in the arena, characterized by significantly increased average velocity as compared to the last habituation day. This was not the case.

Perhaps the most convincing argument for spatiality in NOR is that the novel object is recognized as being such because it occupies the *location* of a previously different object. Along these lines, it could be argued that the novel object elicits more exploration from the control animals because their memory of the spatial representation does not include this object; in other words, it is novel because it is novel in a familiar environment. Based on this argument, the muscimol animals might show impairment even if the hippocampus truly is necessary only for spatial tasks because their impaired spatial navigation on the test day prevents them from recognizing the environment and, therefore, from recognizing a change in one specific feature within that environment. This line of reasoning would conclude that the impaired novel object recognition in the presence of hippocampal inactivation is a result of spatial impairment, not a result of

object memory impairment.

One way to circumvent this argument is by modifying the task to make it less spatial. This was the attempt in the Forwood, Winters and Bussey study (2004) and in the Winters *et al.* study (2004), in which a Y-shaped arena was used. The goal was to eliminate any spatial element that an open arena presents by permitting the animal to see only one object at a time in a very spatially restricted context. The rats in the study could only explore an object by entering the arm that housed it, which prevented the rats from observing the other object simultaneously as part of the context. Since the objects were presented in isolation with this Y-shaped arena, Forwood *et al.* (2004) and Winters *et al.* (2004) argued that the spatial component of the task was diminished. They found that there was no significant difference between groups and concluded that the hippocampus is necessary for object recognition memory only when it involves a rich contextual environment or a spatial component. However, one could just as easily argue that the arena that was used presented its own spatial environment, in which case there must be another reason for the unimpaired performance of the hippocampal-lesioned rats. It is possible that the reason these studies did not reveal object recognition impairments had more to do with their experimental design than with the modified testing arena. Additional NOR studies could further address the issue of spatiality by conducting the test session in a contextually different arena (for example, a pattern on the floor, a differently shaped arena, a different odor in the arena, etc). If the control mice exhibit a novel object preference despite the change in the environment, then one could conclude that the control mice recognize a familiar object as such, even if it is presented in a novel environment. In other words, these results would indicate that although the standard task

might have a spatial component, it is not the determining factor in whether or not the novel object is recognized as being such. If this is found to be the case, then the argument that hippocampal-lesioned mice are impaired only because of spatial deficits would be refuted and the question of spatiality in the NOR task would no longer be a relevant issue.

5.4 Extent of Lesion

It has been argued that hippocampal lesions affecting less than 75% of the hippocampus are insufficient to produce an object recognition deficit (Broadbent et al., 2004). Based on histological verifications, the actual area of the hippocampus affected by the current infusion process appears to be relatively small, and yet the impairments exhibited by the muscimol group are undeniable.

Alternatively, it could be argued that the overarching impairments exhibited by the muscimol groups in the different experiments are evidence that the infusion caused more widespread damage than was intended. Histological verifications revealed disruption in the pyramidal cell layer of the CA1 region of the hippocampus and not in outlying regions; however, it is possible that the drug might have affected the function of extrahippocampal structures without producing a visible cellular disruption.

In a 2008 study, Allen *et al.* found that fluorophore-conjugated muscimol molecule (FCM) acts like muscimol in that it both reversibly blocks excitatory synaptic transmission and produces memory impairments observable in behavioral testing. The study also achieved their primary purpose, which was to establish to what degree FCM spreads from its infusion site. Allen *et al*. (2008) found that myelinated fibers of passage act as natural barriers, preventing FCM from spreading more any more than 0.5 – 1.0 mm

from the site of infusion; it seems likely that muscimol would exhibit a similar disbursement. The current infusion process was very similar to that implemented in the Allen *et al*. (2008) study; in both studies, 0.5 µL per site of 1 µg/µL muscimol or FCM was infused. The current study differed from the Allen *et al*. study by infusing into a different region—the hippocampus instead of the dorsomedial prefrontal cortex (dmPFC) or the basolateral nucleus of the amygdala (BLA)—and by infusing at a slightly slower rate: 0.167 µL/min instead of 0.25 µl/min (Allen et al., 2008). The slower infusion rate implemented in the current study should not deter one from concluding that the Allen *et al*. (2008) findings are relevant; after all, a slower infusion would likely lead to less, rather than more, disbursement of the infusion from the target. The Allen *et al.* (2008) study offers significant support for the conclusion that the present infusions most likely did not spread beyond the hippocampus, but future studies could more definitively address this issue by using FCM instead of muscimol; this would enable a more precise observation of which brain structures are impacted by the $GABA_A$ agonist infusion and a more undeniable conclusion of the role of the hippocampus.

It would also be possible to address this issue by implementing the same infusion process in an established hippocampal-independent task; for example, the mice could be trained and tested in a cue-navigation (visible platform), rather than a place-navigation (submerged platform), version of the Morris water maze paradigm (Morris et al., 1982). If the muscimol mice were impaired in this task, then this result would indicate that the intrahippocampal muscimol infusion also caused extrahippocampal inactivation. In contrast, if the muscimol mice were unimpaired at the hippocampal-independent task, then this result would help to confirm that the present infusion methods resulted in

deficits only in hippocampal-dependent tasks. Such findings would support the conclusion that the current surgical procedure, drug, infusion technique and wait-time between infusion and testing were all effective at preferentially impairing the function of the rodent hippocampus.

5.5 Familiarity vs. Recollection

The current study also speaks to the debate regarding the roles of familiarity and recollection in object recognition, and their respective dependence on the hippocampus. Several studies have examined the differences between recollection and familiarity in rodents.

In a 2004 study, Fortin *et al.* utilized an odor recognition memory task and ROCs to confirm that the hippocampus is integral specifically in recollection memory. Winters and Bussey's 2005 finding that pre-sample, pre-test and time-dependent post-sample lidocaine infusions into the perirhinal cortex of rats disrupts object recognition provide evidence that object recognition is a perirhinal cortex dependent task of familiarity (Winters & Bussey, 2005). Furthermore, studies have demonstrated that rats with hippocampal lesions are impaired in object recognition tasks only when the sample and test sessions occur in different contexts (Good, Barnes, Staal, McGregor, & Honey, 2007; O'Brien, Lehmann, Lecluse, & Mumby, 2006); this finding supports the argument that familiarity based object recognition is independent of the hippocampus, and only recollection—accurately retrieving details about the context in which an item was encountered—depends on the integrity of the hippocampus. Likewise, a Sauvage *et al*. (2008) study utilized odor-medium pairs to demonstrate that the hippocampus supports recollection but not familiarity.

In the current study, inactivation of the dorsal hippocampus impaired object recognition, supporting the idea that this task is a recollection task; however, simply considering the experimental design it seems that it would be appropriate to consider the task one of familiarity. It is not necessary for the mice to recall the specific details or context in which they encountered the object to recognize it as being familiar. One would expect that the mere familiarity of the object gained by prior exploration would be sufficient to produce a novel object preference. These results indicate that either the task is dependent upon recollection in addition to familiarity or that familiarity also depends on unimpaired hippocampal function. However, if familiarity, alone, is sufficient to produce a novel object preference and if familiarity depends on the perirhinal cortex rather than the hippocampus, then one might argue that the current findings indicate that muscimol infusions likely affected both the hippocampus and the perirhinal cortex. While this is a plausible explanation for the current findings, it is not supported by histological verifications, which confirm accurate and isolated infusion into the dorsal CA1 region of the hippocampus.

Future studies that utilize FCM, as previously discussed, could confirm that muscimol does not diffuse out of the target region. In addition, studies that target the perirhinal cortex instead of the hippocampus with an otherwise identical procedure might help to address this critique. Finally, conducting additional studies that implement a change in context, such as those used in the O'Brien *et al*. (2006) study would also further address this issue. The O'Brien *et al*. (2006) study utilized NMDA infusion into the hippocampus in order to produce permanent lesions and found that only context-dependent object recognition was impaired. By combining the same experimental design

with the current study's method of transient hippocampal inactivation via muscimol, future studies will elucidate not only the relevance of context in hippocampal dependent object recognition but also the effects of permanent versus temporary hippocampal inactivation.

5.6 Time-dependence

The current study also addresses the much debated topic of the time-dependent role of the hippocampus in memory. The graded retrograde amnesia exhibited by patients with hippocampal damage indicates that the hippocampus' role in memory retrieval is time dependent—the logical explanation for the saving of the old memories but the loss of more recent memories is that over time memories are transferred out of the hippocampus, likely into neocortical structures.

Maviel *et al.* (2004) mapped the expression of immediate early genes *zif*268 and c-*fos* in C57BL/6J mice trained in a spatial radial arm maze task. They found that when a test session was administered 30 days later, as opposed to 1 day later, there was a significant increase in Zif268 immunoreactive neurons in the prefrontal and anterior cingulate cortices, indicating that these cortical regions house spatial memories once they become independent of the hippocampus (Maviel et al., 2004). Maviel et al. (2004) also examined the behavioral implications of this immediate early gene expression by using lidocaine to temporarily inactivate the dorsal hippocampus, the posterior cingulate cortex, the prefrontal cortex or the anterior cingulate cortex of mice during a retrieval test either 1 or 30 days after training. They found that inactivation of the hippocampus and the posterior cingulate cortex impaired performance in the recent (1 day delay) testing condition only, while prefrontal and anterior cingulate cortical inactivation impaired

performance on the remote (30 day delay) test (Maviel et al., 2004). These findings indicate that the role of the rodent hippocampus in spatial memory is time-dependent; specifically, it is involved for less than 30 days. Current findings potentially contradict the conclusion of the Maviel *et al.* (2004) study.

One half of the mice used in the current NOR experiment were run in a different NOR experiment approximately 21 days prior, at which time they experienced the same familiar object as that used in the current study. If the memory for the familiar object had become relocated to the prefrontal and anterior cingulate cortices, as would be expected based on the findings in the Maviel *et al*. study, then hippocampal inactivation in the current study would not have revealed impaired novel object recognition; instead, the muscimol mice from this specific cohort would have exhibited a stronger NOPR than the truly naïve muscimol mice, simply based on their first round experience with the novel object. The current study found that the mice that received intrahippocampal muscimol from both cohorts were equally impaired; an independent samples Student's *t*-test revealed that the NOPR of the two subgroups (naïve to sample object prior to test week vs. not naïve to sample object prior to test week) were not significantly different (Figure 31; $t_5 = 0.814$, P = 0.453), indicating that the object recognition memory of the animals with prior familiar-object experience had not been transferred out to neocortical regions—any memory that was retained from the initial round of experiments must have remained hippocampal-dependent.

It is important to note, however, that in Maviel *et al*.'s study the remote condition was 30 days; the delay between NOR studies in this case was only 21 days. Therefore, it is possible that the current findings do not contradict the conclusion of the Maviel *et al*.

study; rather, they might further clarify the time necessary for memory to be transferred to neocortical as greater than 21 days or clarify that although spatial memories are consolidated into neocortical regions, object recognition memories are not. These alternative conclusions seem unlikely, but only additional studies would reveal whether the current study contradicts or refines the conclusions of the Maviel *et al*. study.

5.7 Conclusion

In conclusion, the current study demonstrates that intracranial bilateral dorsal CA1 microinfusions of muscimol effectively impair the retrieval of spatial memory, as exhibited in the Morris water maze experiment, and object recognition memory, as exhibited in the Novel Object Recognition experiment. These findings support the hypothesis that the hippocampus is necessary for object recognition memory retrieval and that lesion size is not necessarily a determining factor for object recognition memory impairment. Furthermore, the current findings indicate that either the novel object recognition task is recollection dependent or that familiarity-based recognition also depends on the integrity of the hippocampus.

The current study answers many questions about the role of the rodent hippocampus in non-spatial memory; however, many questions remain. Future studies could utilize FCM and/or implement an identical infusion protocol with an established hippocampal-independent task in order to establish that the damage incurred in the present study did not exceed the hippocampus. Studies implementing isolated pre-sample session or post-sample session infusions could address whether the rodent hippocampus plays an essential role in the encoding and retrieval of both spatial and non-spatial memories; while studies implementing both pre-sample *and* pre-test infusions could

address the question of state dependency. NOR studies that infuse solely into extrahippocampal structures, such as the entorhinal or perirhinal cortices, could address whether other structures play an equally important role in object recognition, and studies implementing contextually rich NOR environments could distinguish any differences in the role of the hippocampus in contextually-rich object recognition memory. Finally, studies that do not require hippocampal lesions also provide valuable tools for eliciting the role of the hippocampus. Electrophysiological recordings of hippocampal neurons during object recognition tasks and studies like one currently being conducted in the Stackman lab, measuring extracellular glutamate during different stages of object recognition, provide useful, lesion-free methods of determining whether the rodent hippocampus is an active player in object recognition memory.

Much work remains to be done before the role of the rodent hippocampus in learning and memory is clarified. The development of transgenic lines of C57BL/6J mice make them ideal animal models for studies seeking answers about age-related cognitive decline and diseases affecting human learning and memory, such as Alzheimer's disease and Parkinson's Disease. Before results from animal models can be confidently transferred to questions involving humans, the appropriateness of these animal models must first be established. The current study brings these efforts one step closer to fruition.

Novel Object Recognition Studies

Lesion Technique	Extent of Lesion	Time of Lesion	Sample	Delay Between Sample & Test	Result	Citation
Radio-Frequency	~ 70%	Permanent: before sample	30 s obj. exp.	10 s or 1 min	Not Impaired	(Clark, Zola, & Squire, 2000)
Radio-Frequency				10 min, 1 h or 24 h	Impaired	
Ibotenic Acid	~ 90%			10 s or 1 min	Not Impaired	
Ibotenic Acid				10 min, 1 h or 24 h	Impaired	
NMDA	Partial	Permanent: post-sample	5 min x 5 days	1 week	Impaired	(Gaskin, Tremblay, & Mumby, 2003)
				5 weeks	Impaired	
		Permanent: pre-sample	5 min	15 min	Not Impaired	
				24 h	Not Impaired	
Lidocaine	Inactivated	Pre-sample	38 s obj exp	5 min (hpc still off)	Not Impaired	(Hammond, Tull, & Stackman, 2004)
		Pre-sample		24 h	Impaired	
Ibotenic Acid	5 – 30%	Permanent: before sample	15 min fixed	3 h	Not Impaired	(Broadbent, Squire, & Clark, 2004)
	30 – 50%					
	50 – 75%					
	75 – 100%				Impaired	
NMDA	~ 85%	Permanent: before sample	5 min x 5 days	24 h	Not Impaired	(Mumby, Tremblay, Lecluse, & Lehmann, 2005)
	~ 81/5			1 week	Not Impaired	
	~ 88%			3 weeks	Not Impaired	
Ibotenic Acid	Complete	Permanent: before sample	30 s obj. exp.	Varied from 10 s – 24 h	Not Impaired	(Ainge, et al., 2006)
	Partial					
	Complete		3 x 5 min fixed			
	Partial					
Muscimol	Inactivated	Immediately after sample	5 min fixed	1.5 h and 24 h	Impaired	(de Lima, Luft, Roesler, & Schroder, 2006)
		3 h after sample		24 h	Impaired	
		6 h after sample		24 h	Not Impaired	
Muscimol	Inactivated	Pre-test	3 x 10 min	24 h	Impaired	Current Study

Figure 1. Summary of novel object recognition Studies. A brief summary of NOR studies to demonstrate the conflicting findings that have been reported.

Multiple Memory Systems

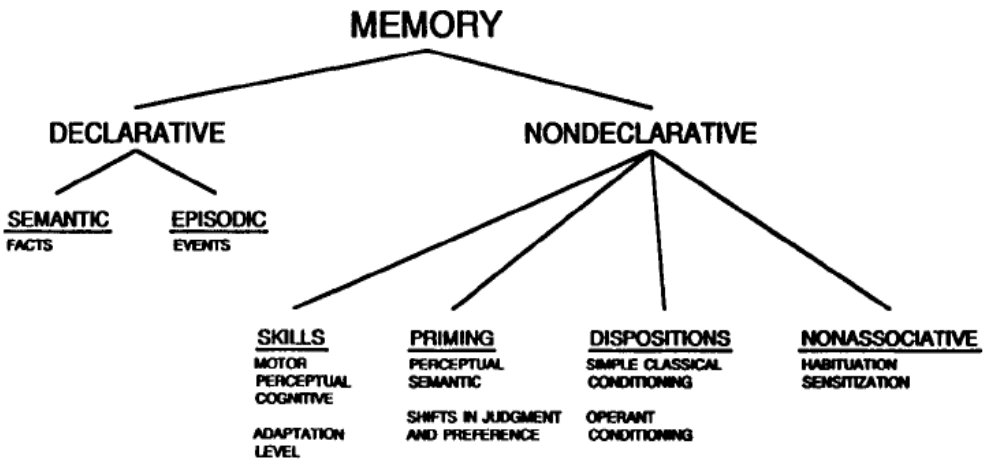


Figure 2. Multiple memory systems. Declarative memory includes semantic memory for facts and episodic memory for events; it is dependent on the hippocampus. Nondeclarative memory encompasses other types of learning, such as skill-learning, priming, conditioning and habituation/sensitization. Nondeclarative memory is independent of the hippocampus (Squire, 1992).

A. The Morris water maze

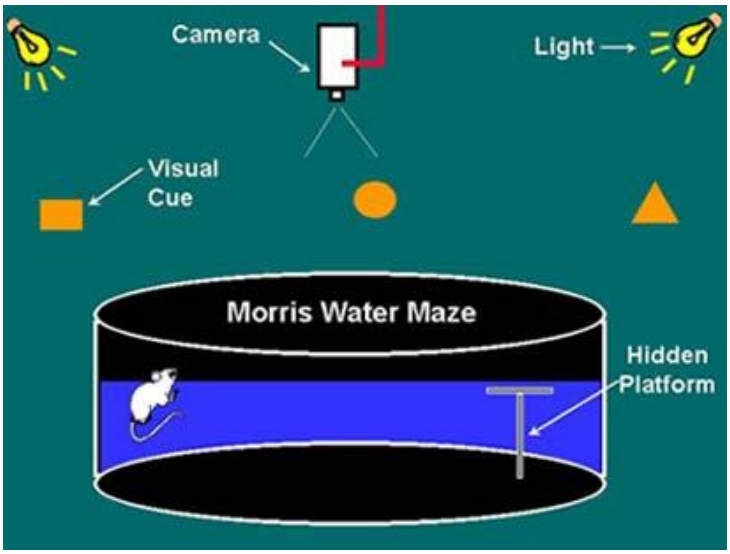


Illustration of MWM retrieved March 20, 2011 from
http://www.georgiahealth.edu/core/labs/sabc/Morriswatermaze.htm

B. Y-shaped modified NOR arena

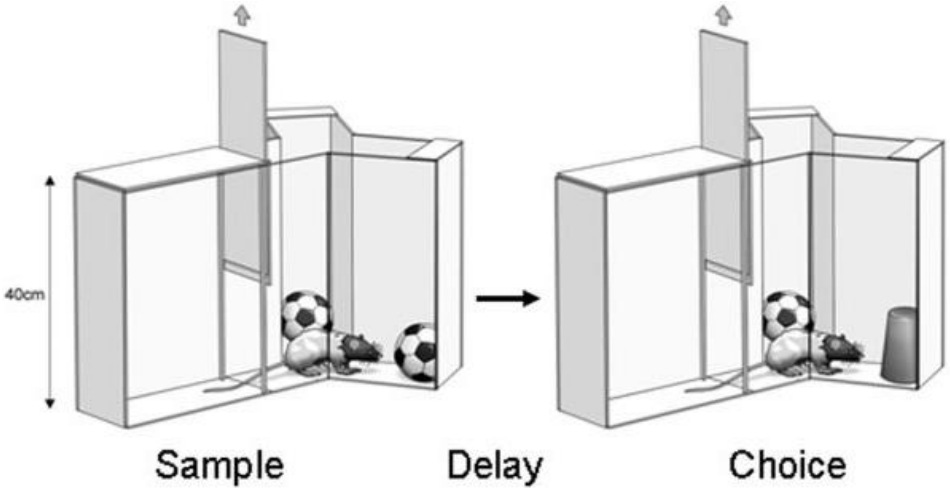
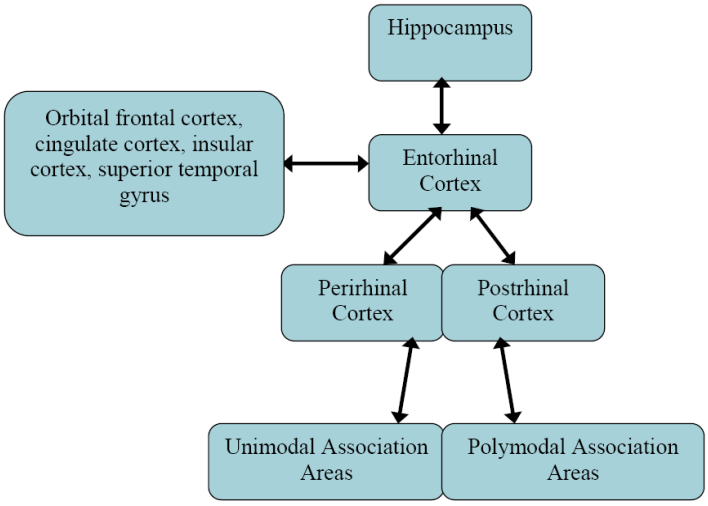


Figure 3. Relevant experiment apparatuses. A. Morris water maze. The standard Morris water maze includes a submerged platform in a pool of opaque water. Extra-maze visual cues are positioned around the room. Over time, the rodent learns to navigate directly to the platform from any starting location. B. Y-shaped modified NOR arena. This Y-shaped NOR arena was used in an effort to eliminate the possible spatial and contextual elements of the standard novel object recognition testing arena. The sample and choice (test) phases of the test are illustrated. The near wall appears transparent for illustrative purposes only (Forwood et al., 2004).

A. Medial Temporal Lobe Projections



B.

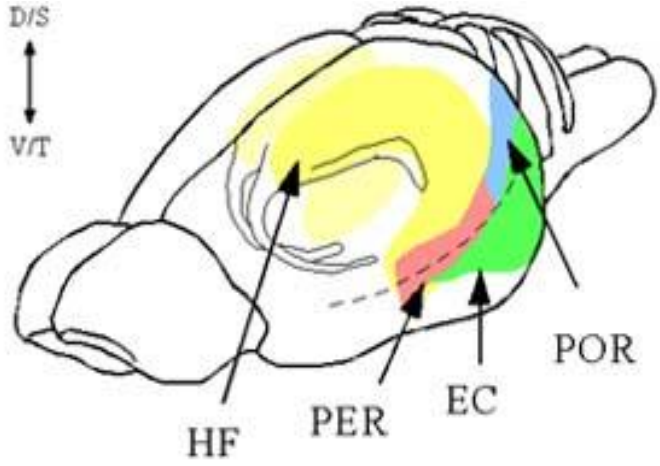


Figure 4. Medial temporal lobe circuits. A. A schematic view of the projections within the medial temporal lobe. Unimodal and polymodal association areas project to the perirhinal and parahippocampal cortices, respectively. The perirhinal and parahippocampal/ postrhinal cortices, along with other brain regions (orbital frontal cortex, cingulate cortex, insular cortex and superior temporal gyrus) project to the entorhinal cortex, which is the main source of inputs to the hippocampus. All projections are reciprocal. B. A schematic view of the medial temporal lobe of the rat brain: the hippocampus (yellow), perirhinal cortex (pink), postrhinal cortex (blue) and entorhinal cortex (green). (Image B. retrieved March 18, 2011 from http://www.brown.edu/Research/Burwell-Lab/Research/Hippocampal_System.jpg.)

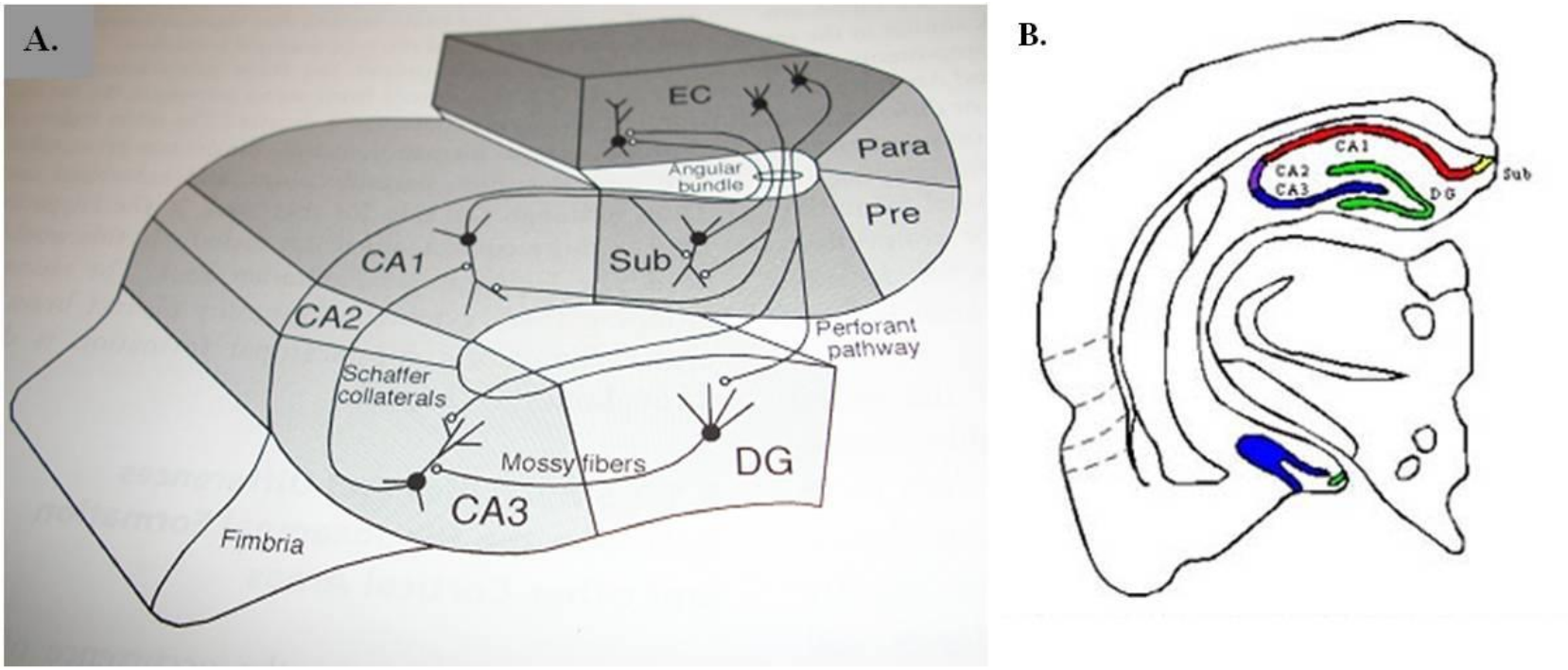


Figure 5. The hippocampal circuit. A. A schematic view of the hippocampal circuit. Superficial layers of the entorhinal cortex project to the dentate gyrus via the perforant pathway. The granule cells of the dentate gyrus project their axons (mossy fibers) to the pyramidal cells of CA3. CA3 sends Schaffer collateral axons to CA1. CA1 pyramidal neurons project to the subiculum and also send reciprocated projections to the entorhinal cortex. The subiculum projects to the presubiculum and parasubiculum. B. A coronal section of a mouse brain.

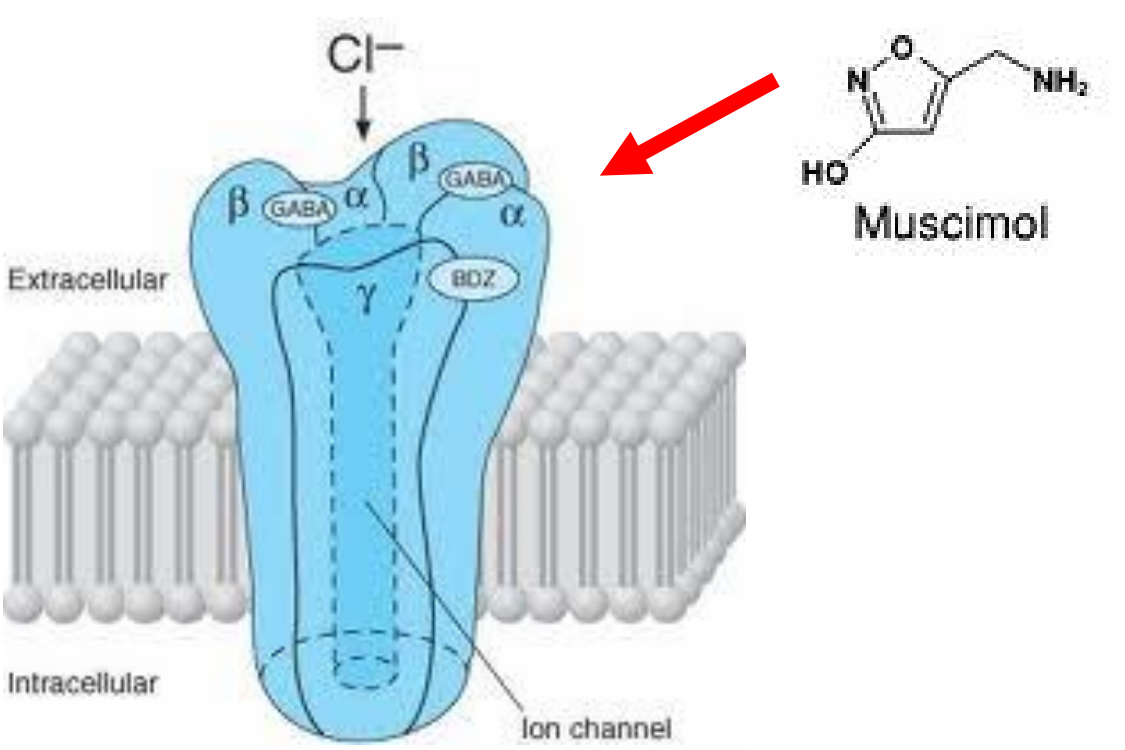


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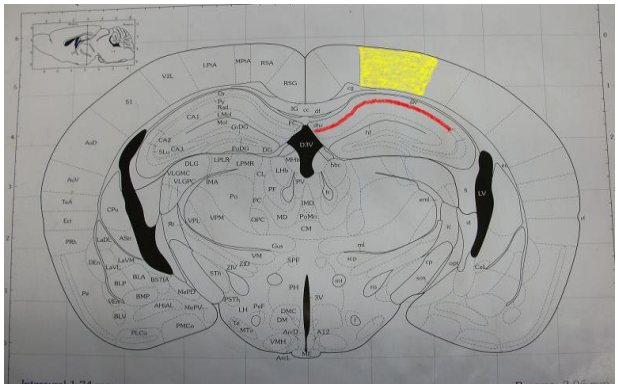
Figure 6. The GABAA receptor. The $GABA_A$ receptor is an ionotropic, heteropentameric receptor that is located on the cell bodies and proximal dendrites of pyramidal cells in the hippocampus. When two GABA molecules bind to the receptor, the Cl^- channel opens, resulting in hyperpolarization. Muscimol, a $GABA_A$ agonist, has the same effect.

A.



B.

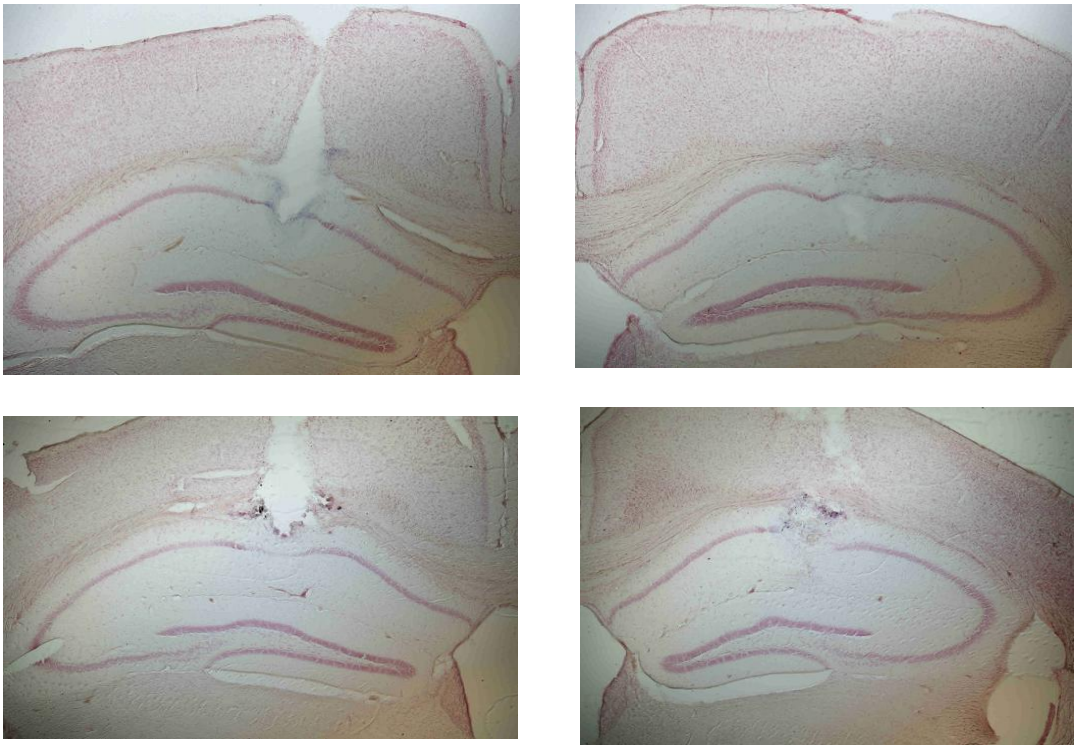


Figure 7. Histological analysis of cannulae placement above dorsal CA1. **A.** Schematic drawing of mouse brain taken from Franklin and Paxinos (2007). Pyramidal cell layer of dCA1 has been marked in red; yellow represents parietal association cortex potentially affected by cannulae placement. **B.** Representative samples of bilateral cannulae placement observed in Neutral Red-stained 50 µm coronal sections of brain tissue from two different mice. Prior to sacrificing the mice, 0.5 µL of Cresyl violet was infused into each side to aid in histological verification.

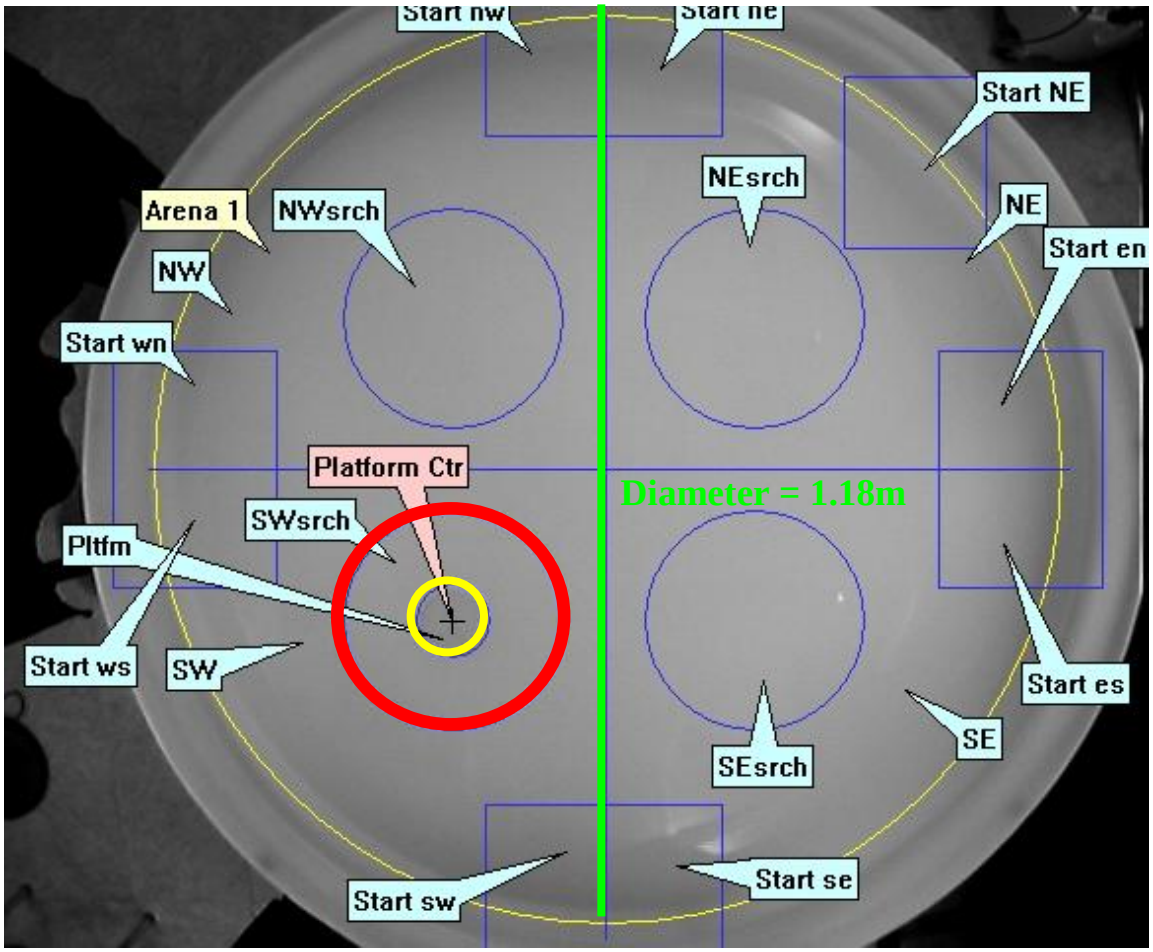


Figure 8. The MWM testing arena and zone definitions. Mice were given 4 training trials per day, one from each start location (N, E, S, W), for each of the 4 training days. During training the platform was always located in the SW quadrant. On test day, the platform was removed. Time spent within the exact platform location (yellow circle) was used to calculate the RIOS Platform:Pool Ratio. The red circle outlines the SW search zone; time spent in this zone was used to calculate the SW Search Zone Ratio.

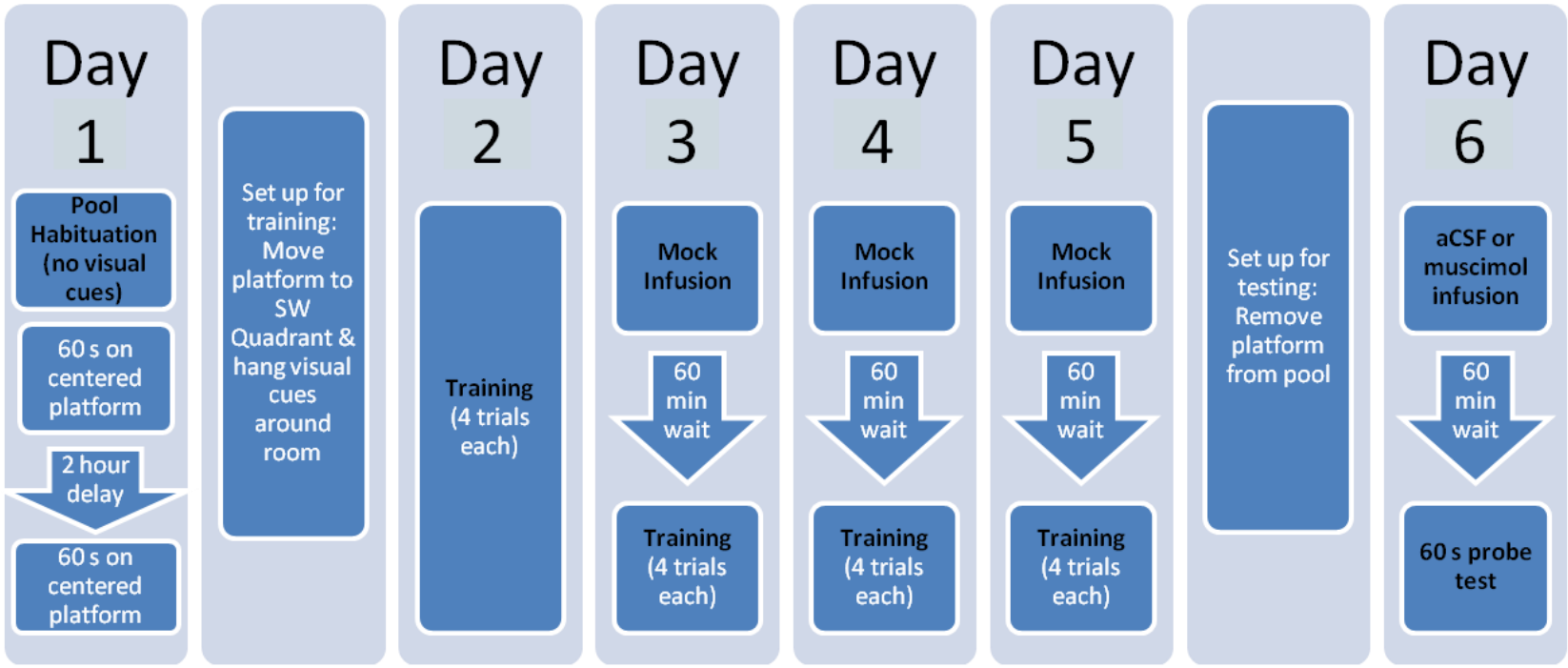


Figure 9. MWM experimental design. On Day 1, mice were given two pool habituation sessions each. During the habituation sessions, the platform was located in the center of the pool and the black curtains around the pool were closed to eliminate any visual cues. On Days 2, 3 and 4, mice were given 4 trials per day, one from each starting point. On Day 5 the platform was removed from the pool and each mouse was given a 60 s probe test.

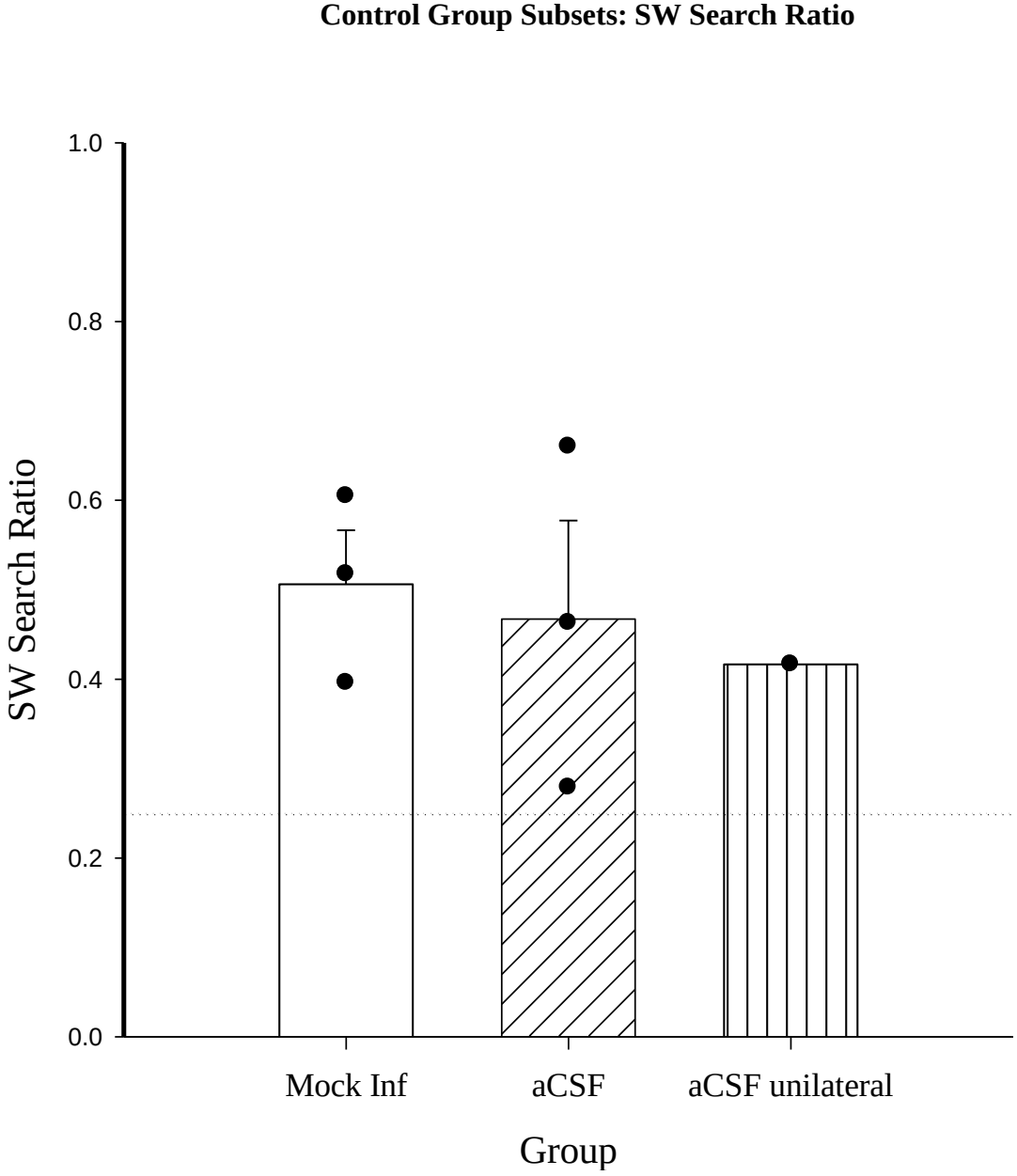


Figure 10. Comparison of SW search ratios of MWM control group subsets. Three mice received mock infusions, three received bilateral aCSF microinfusions and one mouse received a unilateral aCSF microinfusion. There was no significant difference in mean (± S.E.M.) SW search ratio between any of the three subsets ($F_{2,6} = 0.136$, $P = 0.877$). Therefore, subsets were combined into one control group.

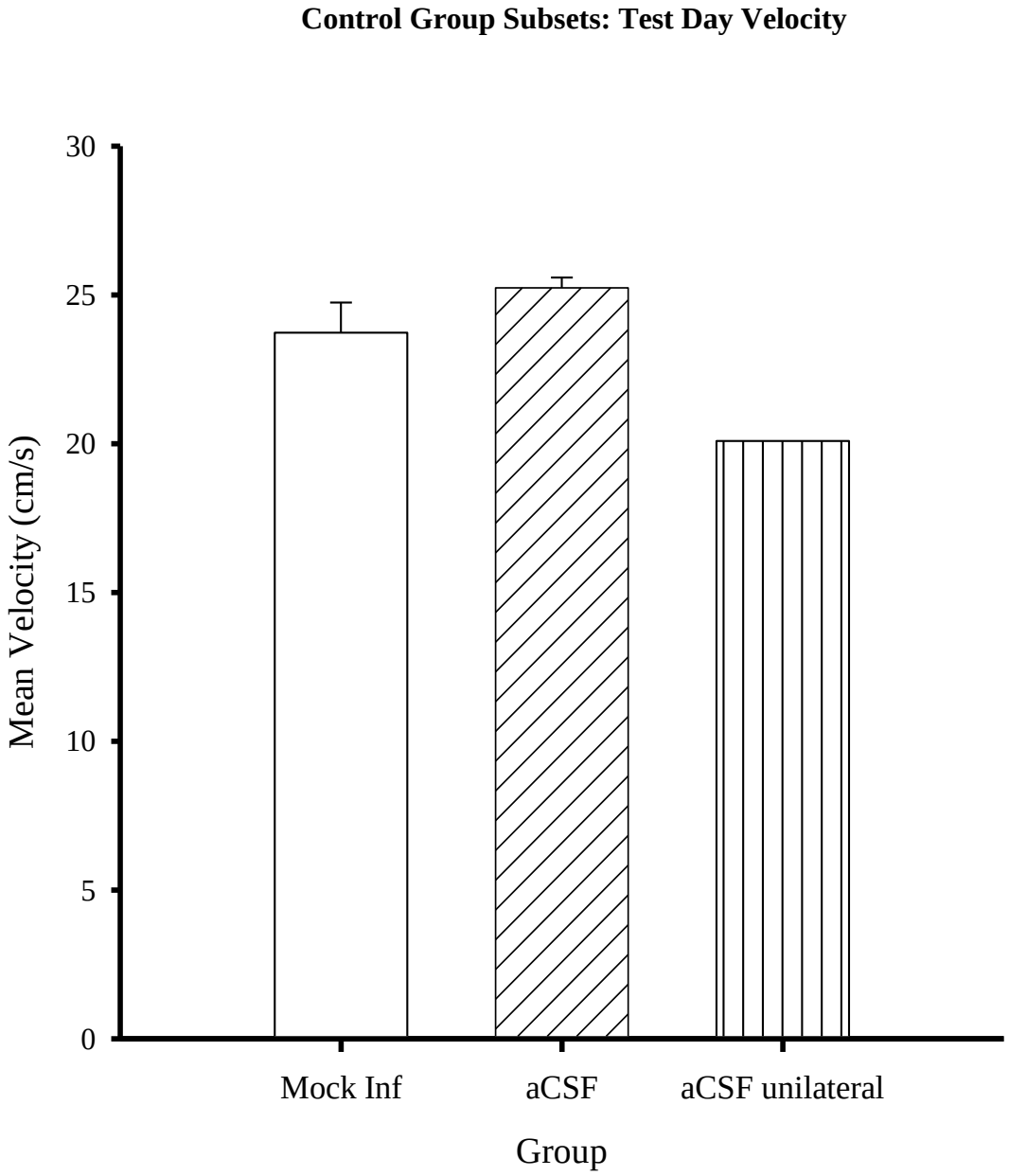


Figure 11. Comparison of test day velocities of MWM control group subsets'. Three mice received mock infusions, three received bilateral aCSF microinfusions and one mouse received a unilateral aCSF microinfusion.There was no significant difference in mean (± S.E.M.) test day velocity between any of the three subsets ($F_{2,6}$ = 5.801, P = 0.066). Therefore, subsets were combined into one control group.

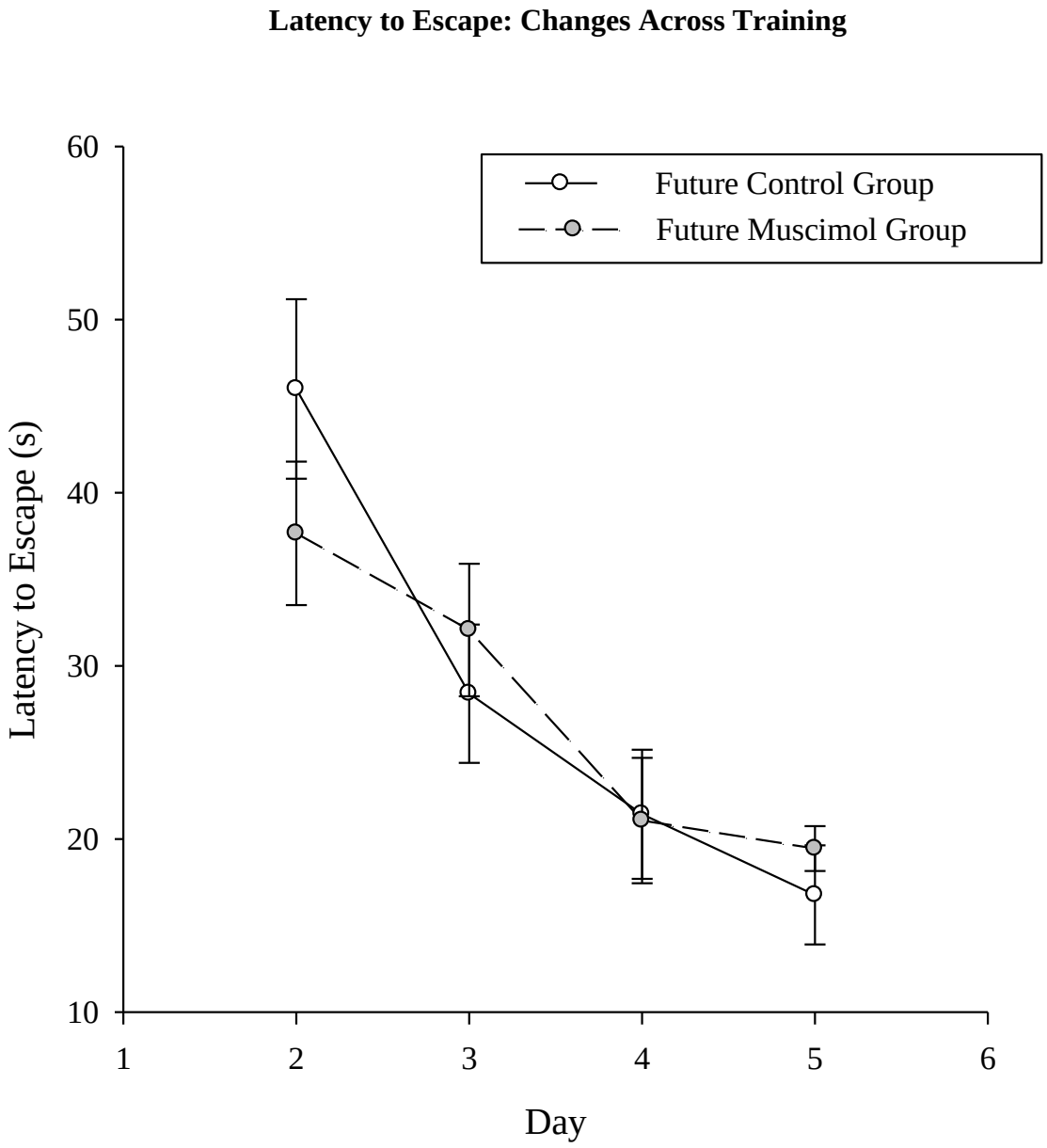


Figure 12. Latency to escape onto the platform during training. Over the 4 days of water maze training, mean latency to escape (± S.E.M.) significantly decreased ($F_{3, 39}$ = 17.863, P < 0.001). There was not a significant difference between future treatment groups ($F_{1, 13}$ = 0.002, P = 0.962), indicating that the future groups both acquired the task equally well. (Note: microinfusions were not administered on any of the training days.)

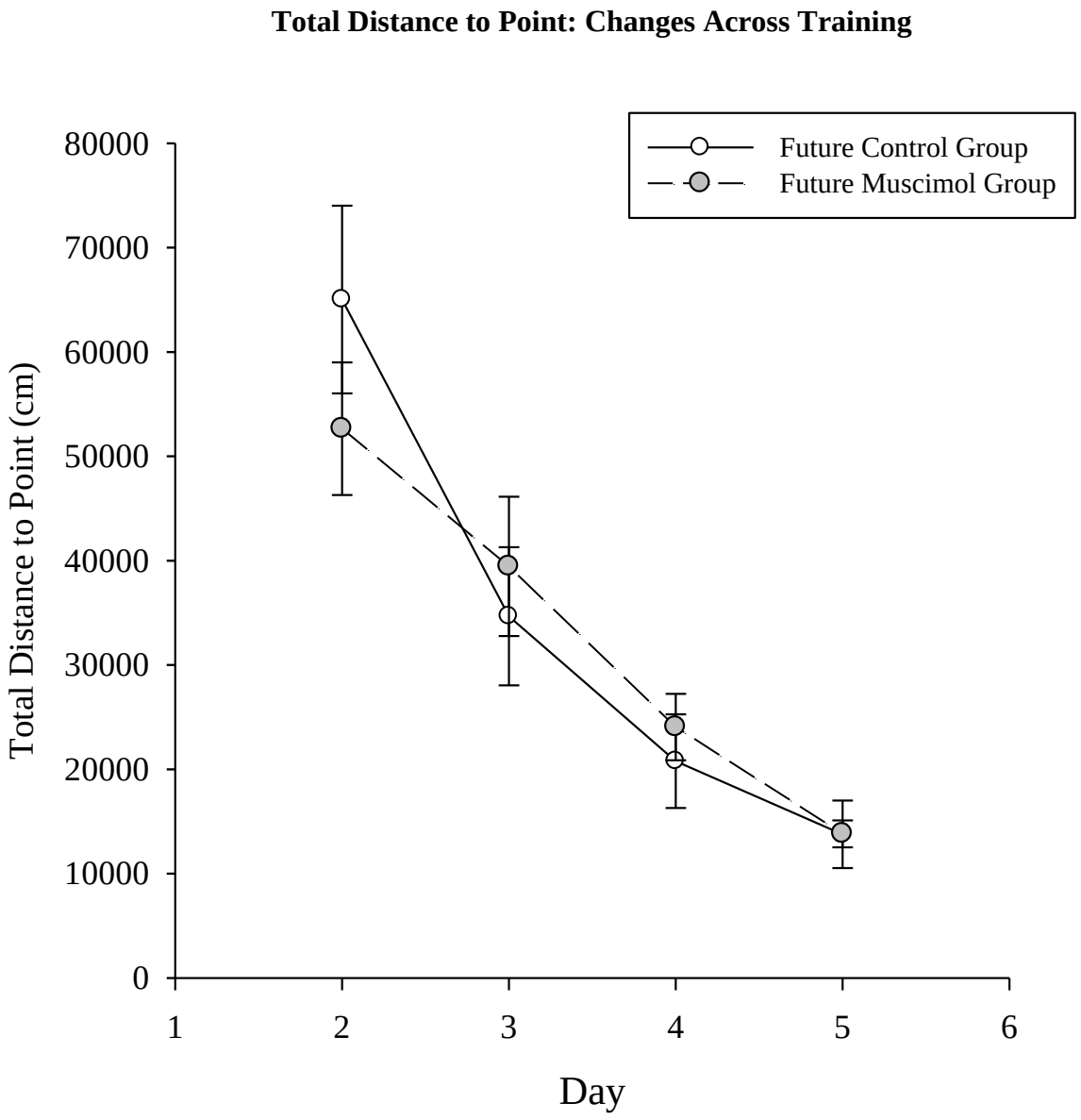


Figure 13. Total distance to the platform center during training. Over the 4 days of water maze training, mean total distance to the platform center (± S.E.M.) significantly decreased ($F_{3, 39} = 28.158$, $P < 0.001$). There was not a significant difference between future treatment groups ($F_{1, 13} = 0.057$, $P = 0.815$), indicating that the future groups both acquired the task equally well. (Note: microinfusions were not administered on any of the training days.)

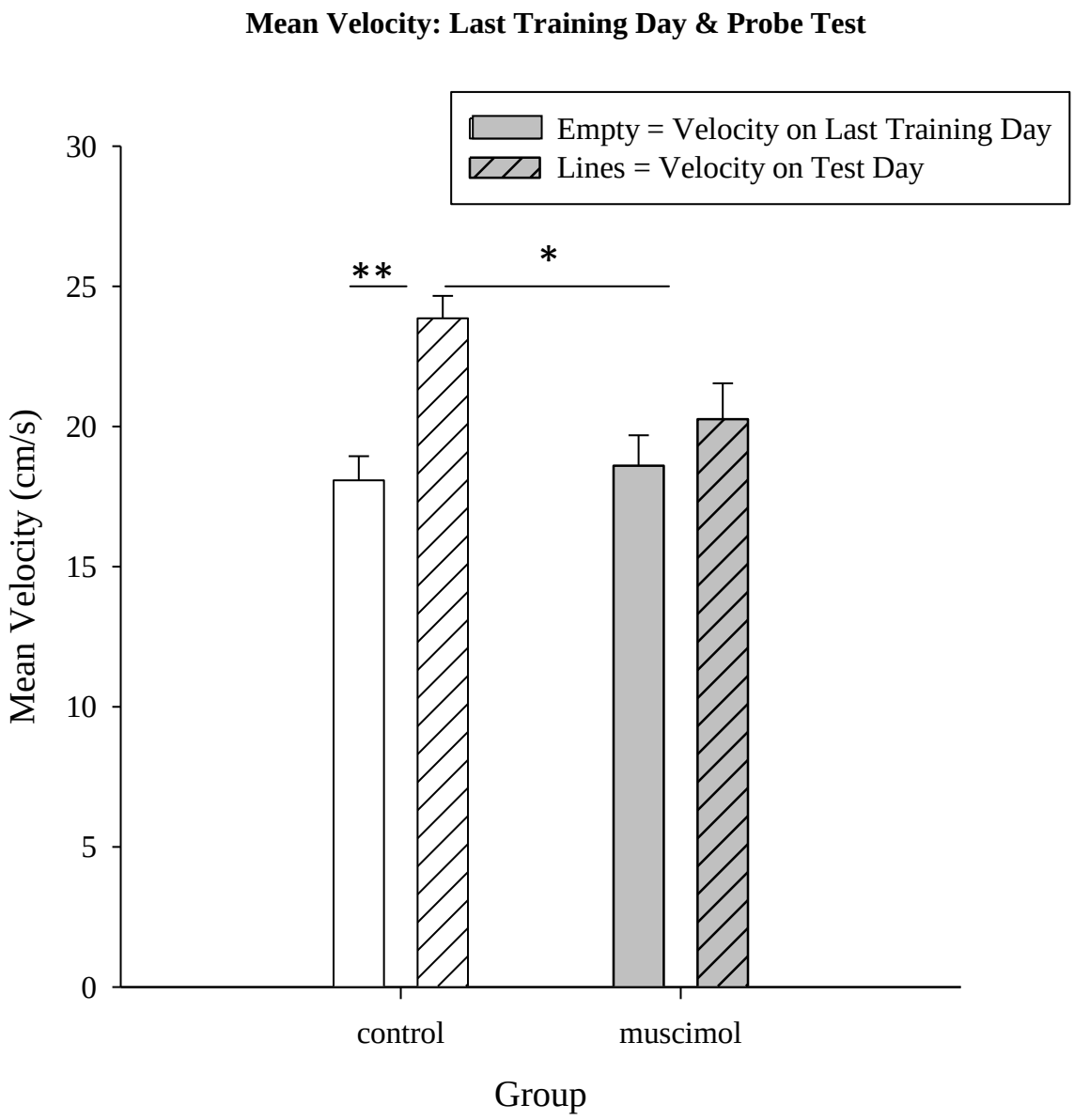


Figure 14. Swim velocity on the last training day and on test day. On the last day of water maze training, the mean swim velocities (± S.E.M.) of the future treatment groups were similar (t_{13} = -0.368, P = 0.719), further verifying that prior to test day infusions the performance of both groups were equal. The control group's swim velocity unexpectedly and significantly increased from the last training day to the test day (t_7 = -9.053, P < 0.001), while the muscimol group's swim velocity stayed approximately the same as the day before. On test day, the velocity of the control group was significantly higher than that of the muscimol group (t_{13} = 2.297, P = 0.039); this appears to be a result of the control group's increase in speed, and not any indication that the muscimol groups' swimming ability was affected by the muscimol microinfusion. (** = P < 0.001; * = P < 0.05)

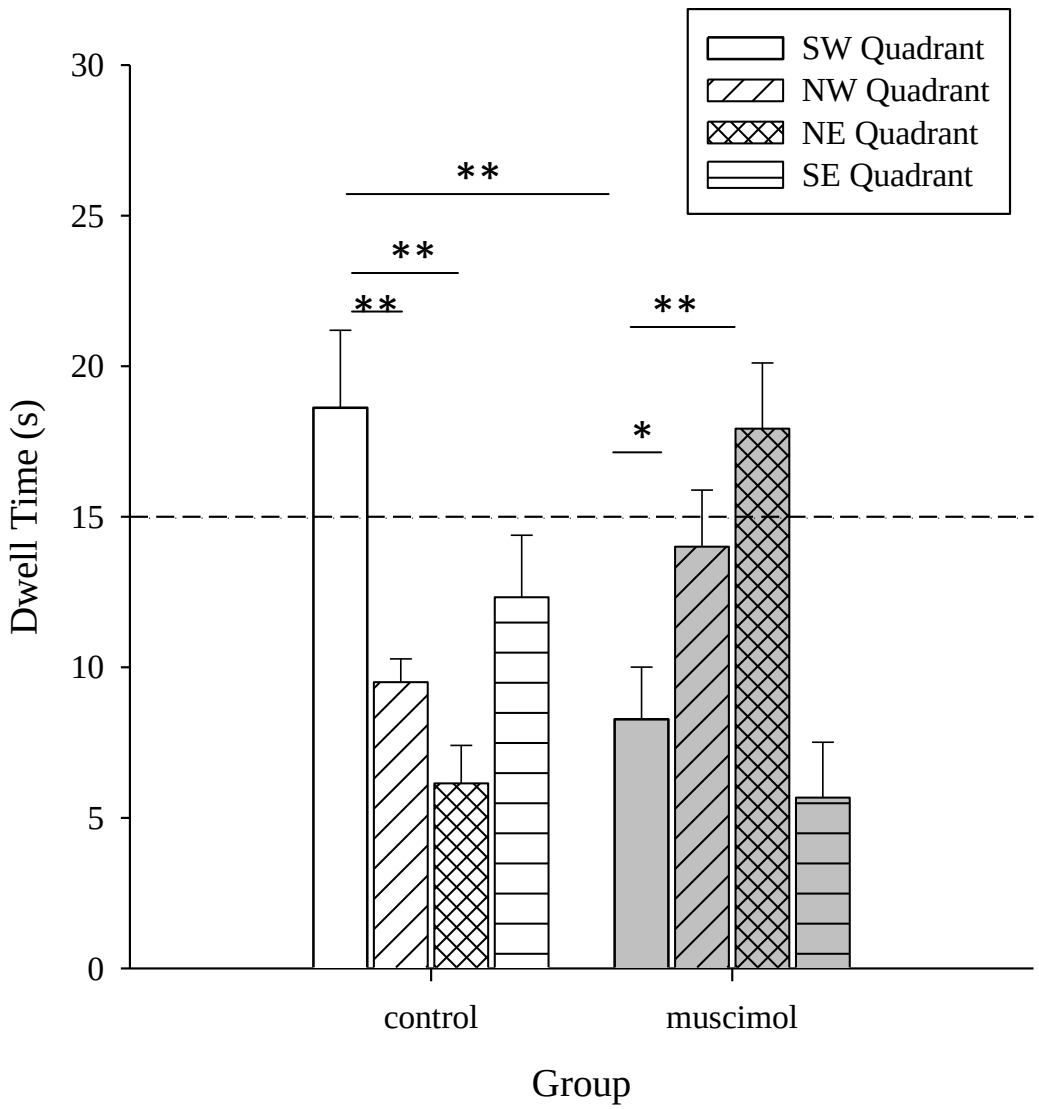


Figure 15. MWM test day quadrant dwell times. Significant differences related to the mean (± S.E.M.) SW quadrant dwell times are indicated above. Most importantly, the control group spent significantly more time in the SW quadrant than did the muscimol group (t_{13} = 3.986, P = 0.002). Chance (15 s) is indicated by the dashed line. (** = P < 0.01; * = P < 0.05)

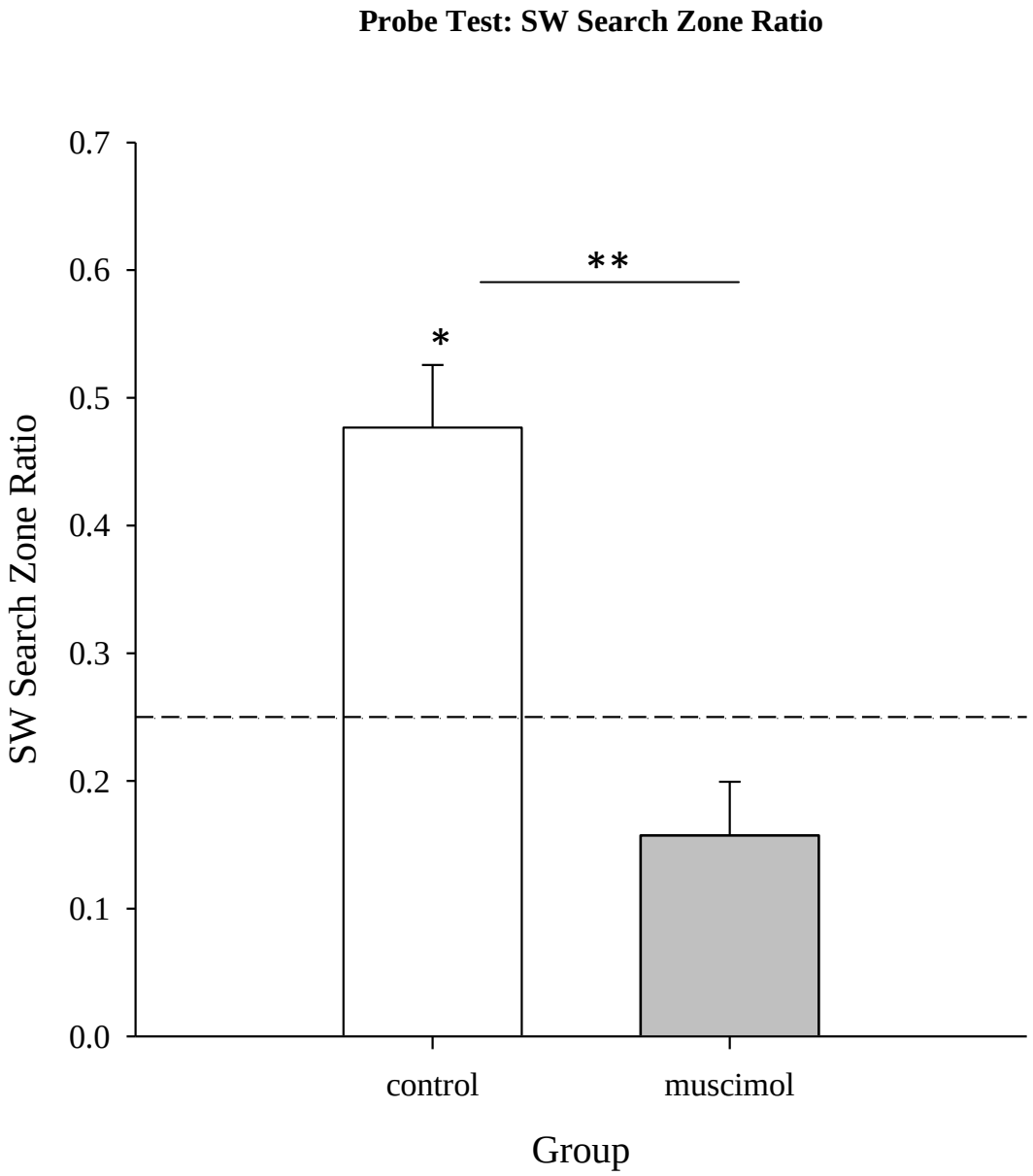


Figure 16. MWM test day SW search zone ratios for each group. SW search zone ratio is defined as the amount of time spent in the SW search zone divided by total time spent in any of the 4 search zones (see figure 9). The control group's mean (± S.E.M.) SW search ratio was significantly greater than chance ($t_6 = 4.619$, P = 0.004, *) and significantly greater than the muscimol group's SW search ratio ($t_{13} = 4.973$, P < 0.001, **), which was less than chance, but not significantly ($t_7 = 2.204$, P = 0.063). Chance (0.25) is indicated by the dashed line.

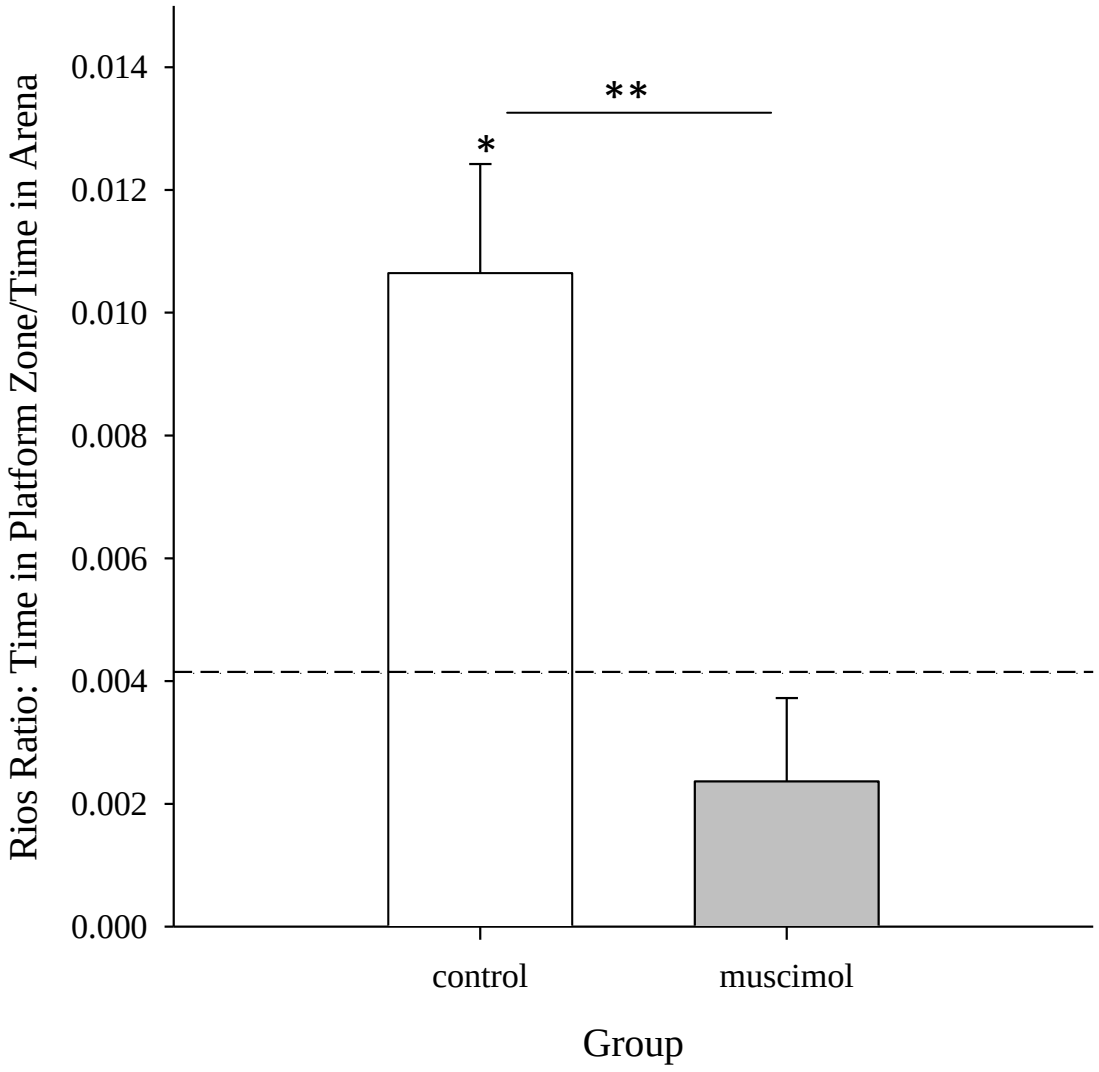


Figure 17. MWM test day RIOS platform:pool ratios for each group. RIOS (real instances of success) platform:pool ratio is defined as the amount of time spent in the target platform zone divided by total time spent in the Morris water maze (see figure 9). The control group's mean (± S.E.M.) RIOS platform:pool ratio was significantly greater than chance (t_6 = 5.952, P = 0.001, *) and significantly greater than the muscimol group's mean (± S.E.M.) RIOS platform:pool ratio (t_{13} = 3.758, P = 0.002, **), which not significantly different from chance (t_7 = 1.691, P = 0.135). Chance (0.004148) is indicated by the dashed line.

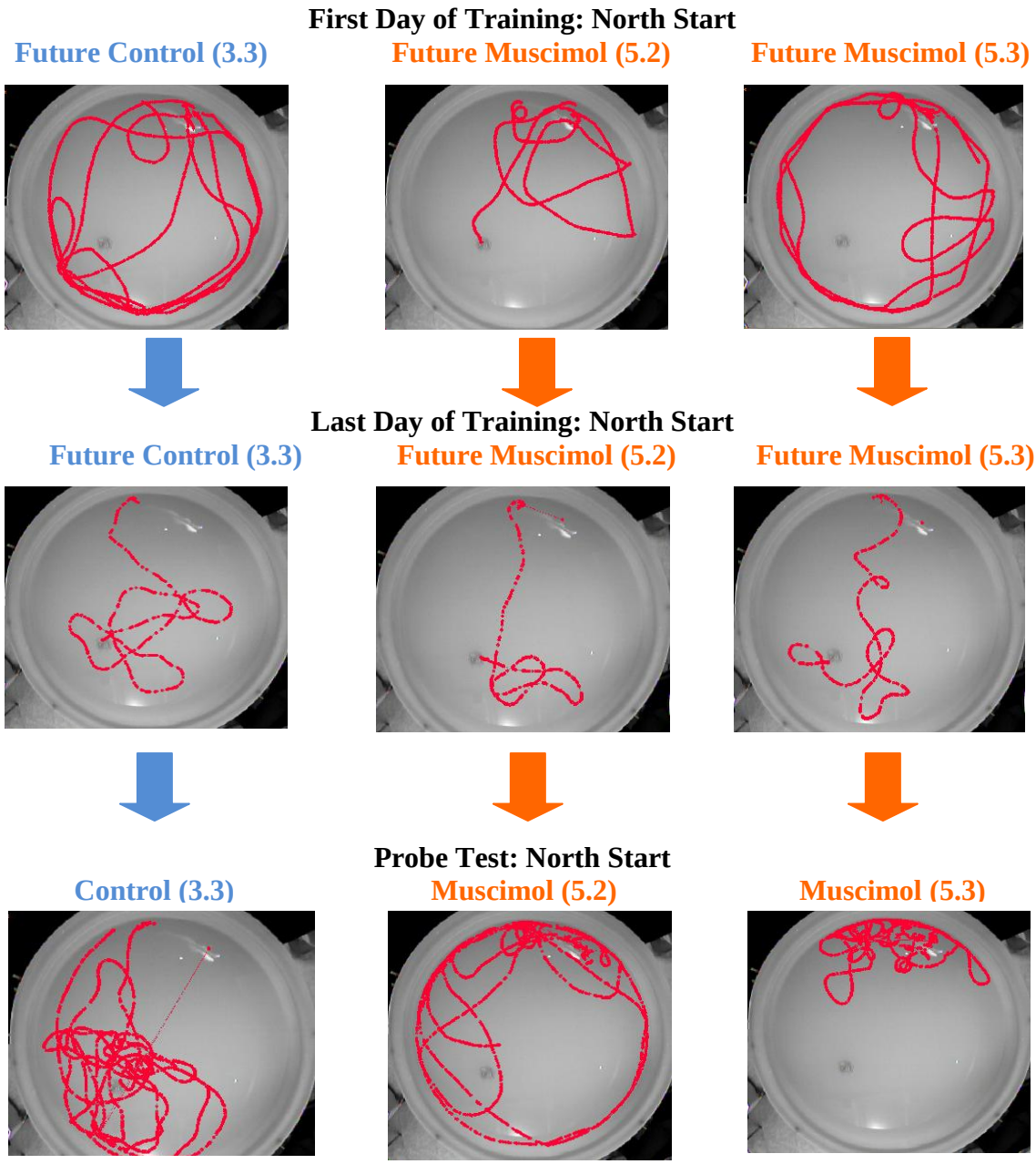


Figure 18. Representative swim paths. Swim paths for 1 control mouse and 2 muscimol mice are shown above. On the first day of water maze training (first row) none of the mice demonstrated effective search strategies. On the last day of training (middle row) all mice showed dramatic improvement. On the probe test (last row) the control mouse demonstrated concentrated searching in the correct location, while the muscimol mice did not, as illustrated by the respective swim paths. Note that the searching strategies of the muscimol mice on test day are similar or worse than their searching strategies on the first training day.

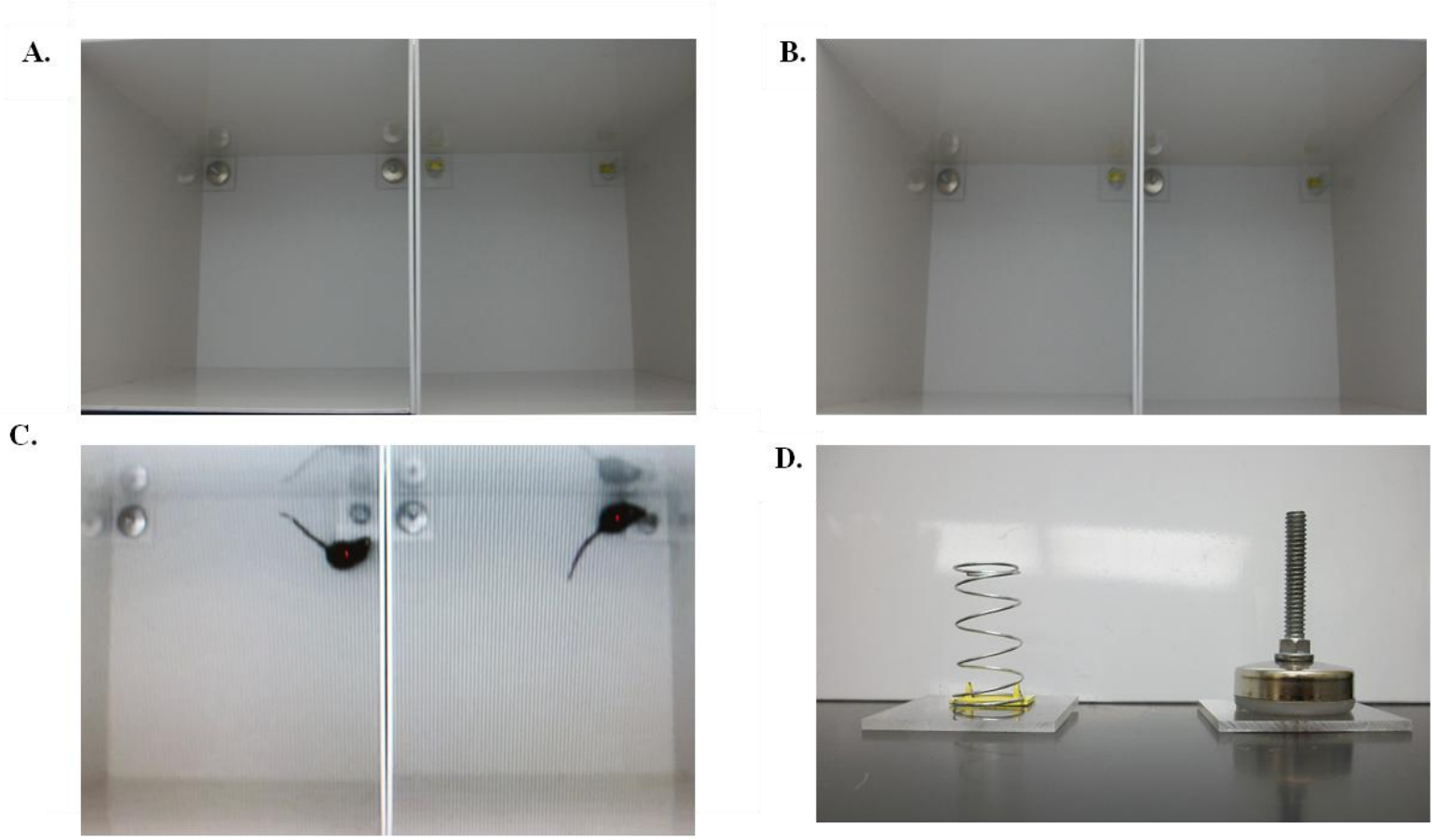


Figure 19. The NOR objects and testing arena. A. Sample session arena setup. Each arena contains two identical objects, one in the NW corner and one in the NE corner. B. Test session arena setup. Each arena contains two different objects—one familiar and one novel. The objects used in each arena, the object used as novel and the location of the novel object were all counterbalanced across trials. C. Image of animals exploring objects during a test session. D. Objects: spring (left) and foot (right).

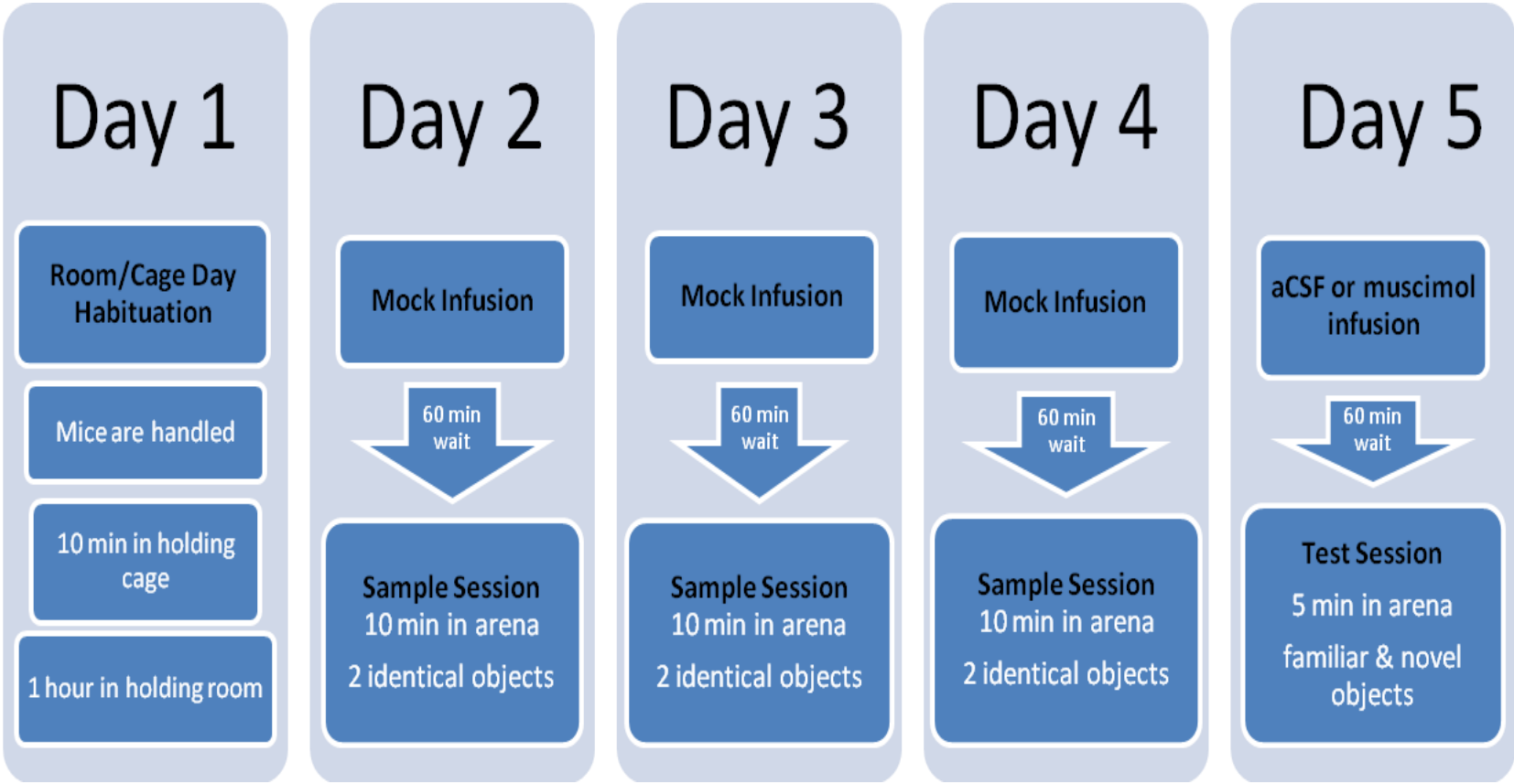


Figure 20. NOR experimental design. On Day 1, mice were habituated to being handled, to being in the polycarbonate holding cages and to being in the holding room. On Days 2, 3 and 4 mice received mock infusion and were then placed in holding cages. After 60 min, mice were placed in the NOR arena and given 10 min to explore the arena and the objects. On Day 5, mice received actual infusions, were placed in holding cages for 60 min then given a test session, during which one familiar object had been replaced with a novel object.

NOR Preliminary Studies: Analysis of Zone Preferences

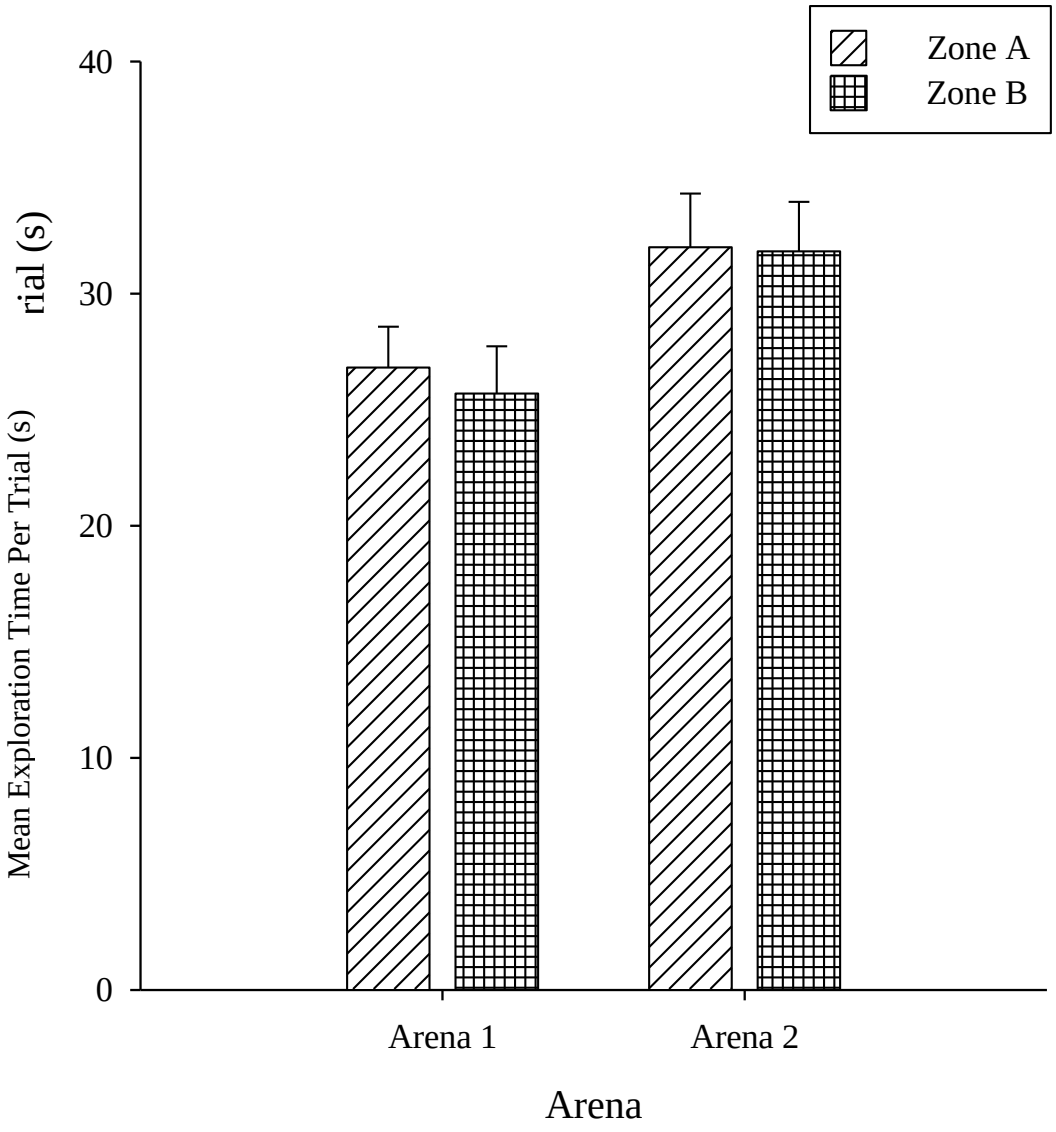


Figure 21. NOR preliminary zone preference testing. Preliminary studies indicated that the arena zones themselves did not elicit any preference. In both arenas, the amount of object exploration in zone A was not significantly different than the amount of object exploration in zone B. This was true in Arena 1 (t_{34} = 0.643, P = 0.524) and in Arena 2 (t_{34} = 0.058, P = 0.954).

NOR Preliminary Studies: Novel Object Preferences

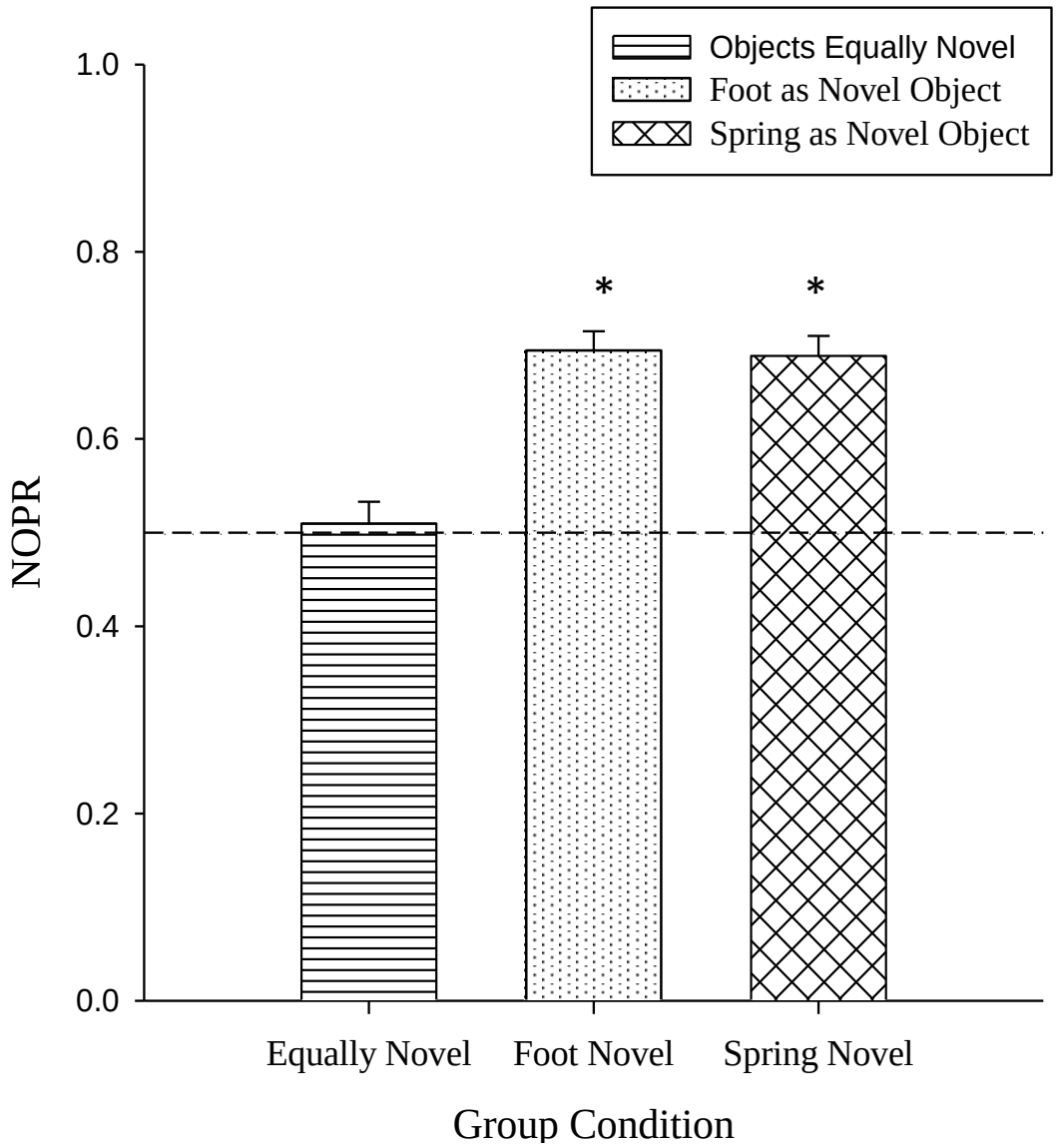


Figure 22. NOR preliminary object preference testing. Preliminary studies revealed that when both objects were equally novel, there was not a significant preference for one over the other ($t_7 = 0.410$, P = 0.694). However, when the foot was the novel object, it did elicit a novel object preference ratio significantly greater than chance ($t_7 = 9.514$, P < 0.001, *), as did the spring when it was novel ($t_7 = 8.760$, P < 0.001, *).

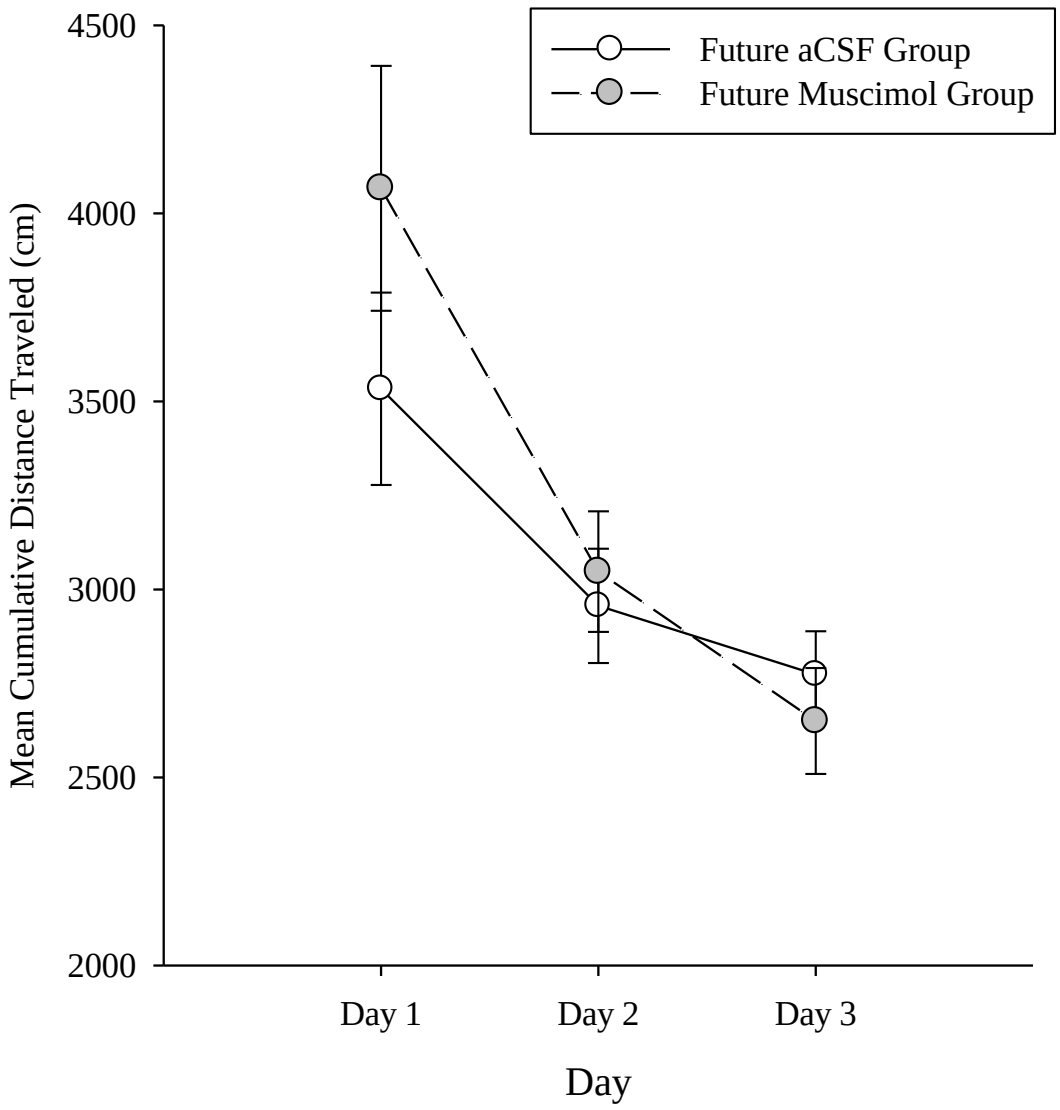


Figure 23. NOR cumulative distance traveled across training. There was a significant decrease in the mean (± S.E.M.) cumulative distance traveled from one training day to the next ($F_{1, 15} = 84.829$, $P < 0.001$); however, there was not a significant difference between future groups on any of the training days ($F_{1, 15} = 1.878$, $P = 0.191$). These results indicate that prior to test day infusions, the two future groups exhibited similar locomotion within the arenas.

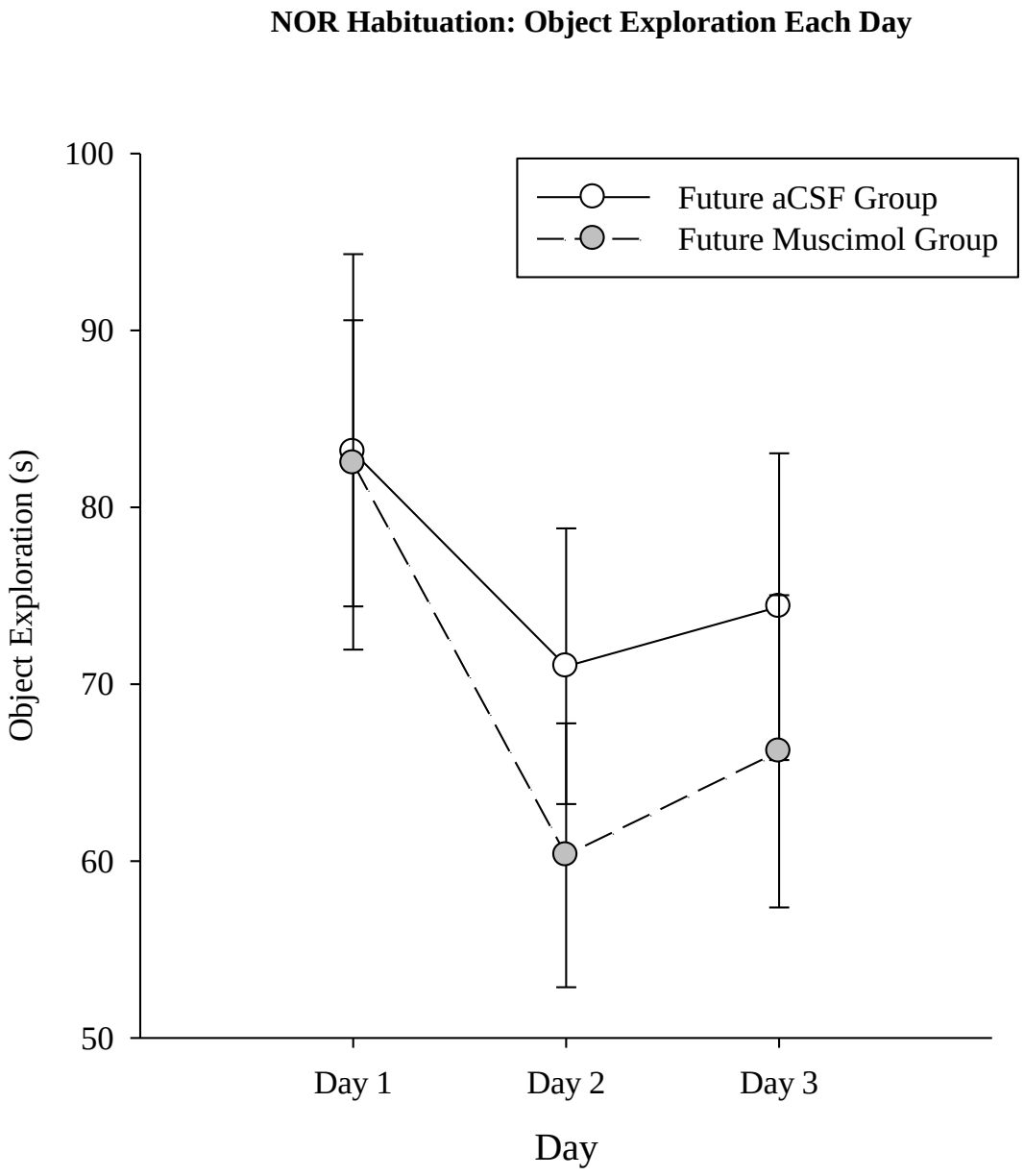


Figure 24. NOR object exploration across training. There was not a significant difference in mean (± S.E.M.) object exploration across days ($F_{1, 15} = 2.955$, $P = 0.106$) or between future groups ($F_{1, 15} = 0.377$, $P = 0.548$). These results indicate that prior to test day infusions the future groups exhibited similar object exploration.

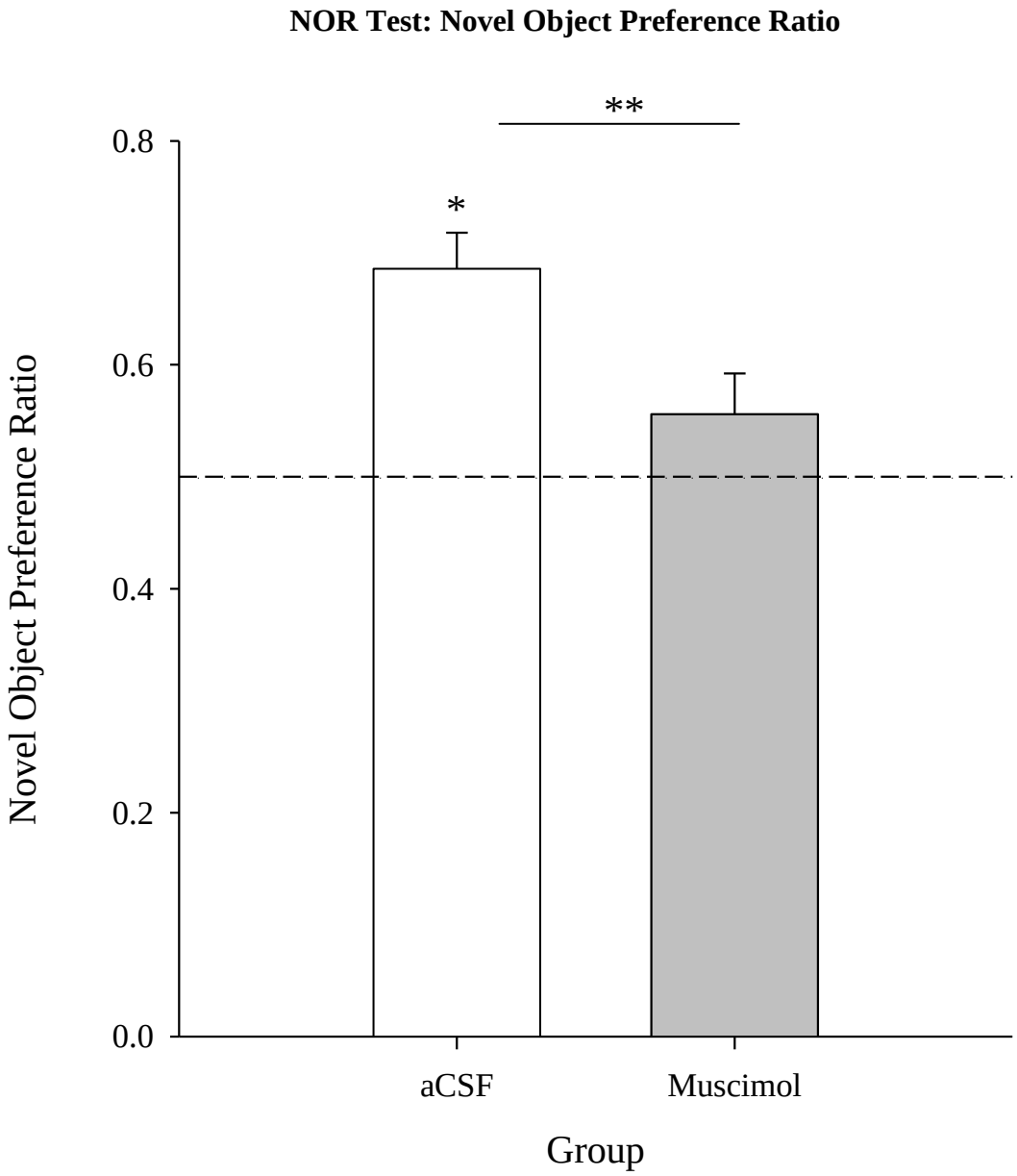


Figure 25. NOR test day novel object preference ratios. On test day, the aCSF group exhibited a mean (± S.E.M.) novel object preference ratio (NOPR) that was significantly greater than chance ($t_9 = 5.745$, P < 0.001, *) and significantly greater than the mean (± S.E.M.) NOPR of the muscimol group ($t_{15} = 2.640$, P = 0.019, **). The NOPR of the muscimol group was not significantly different than chance ($t_6 = 1.532$, P = 0.176).

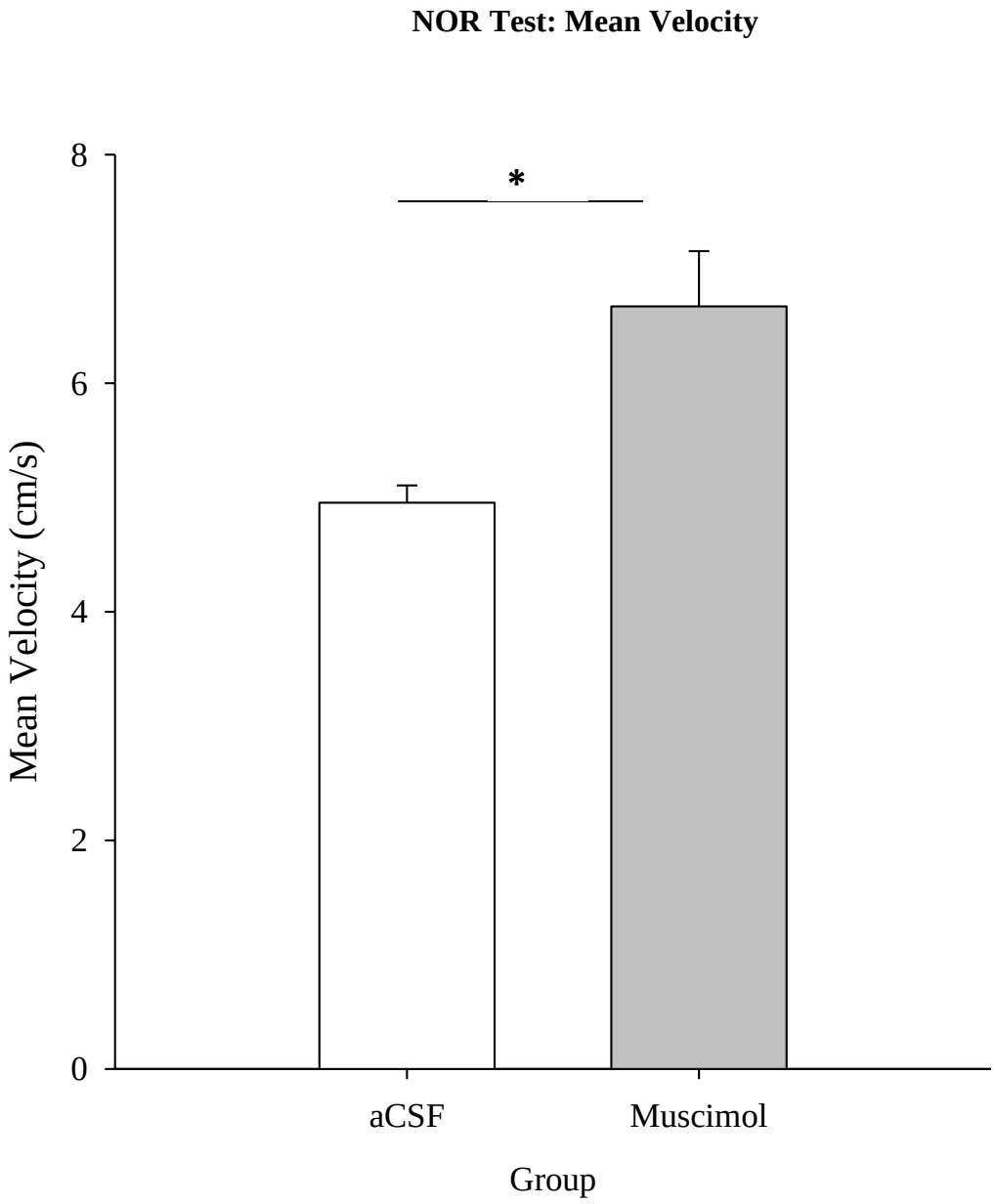


Figure 26. NOR test day velocities. The mean (± S.E.M.) velocity of the muscimol group on test day was significantly greater than the mean (± S.E.M.) velocity of the aCSF group (t_{15} = -3.918, P = 0.001, *).

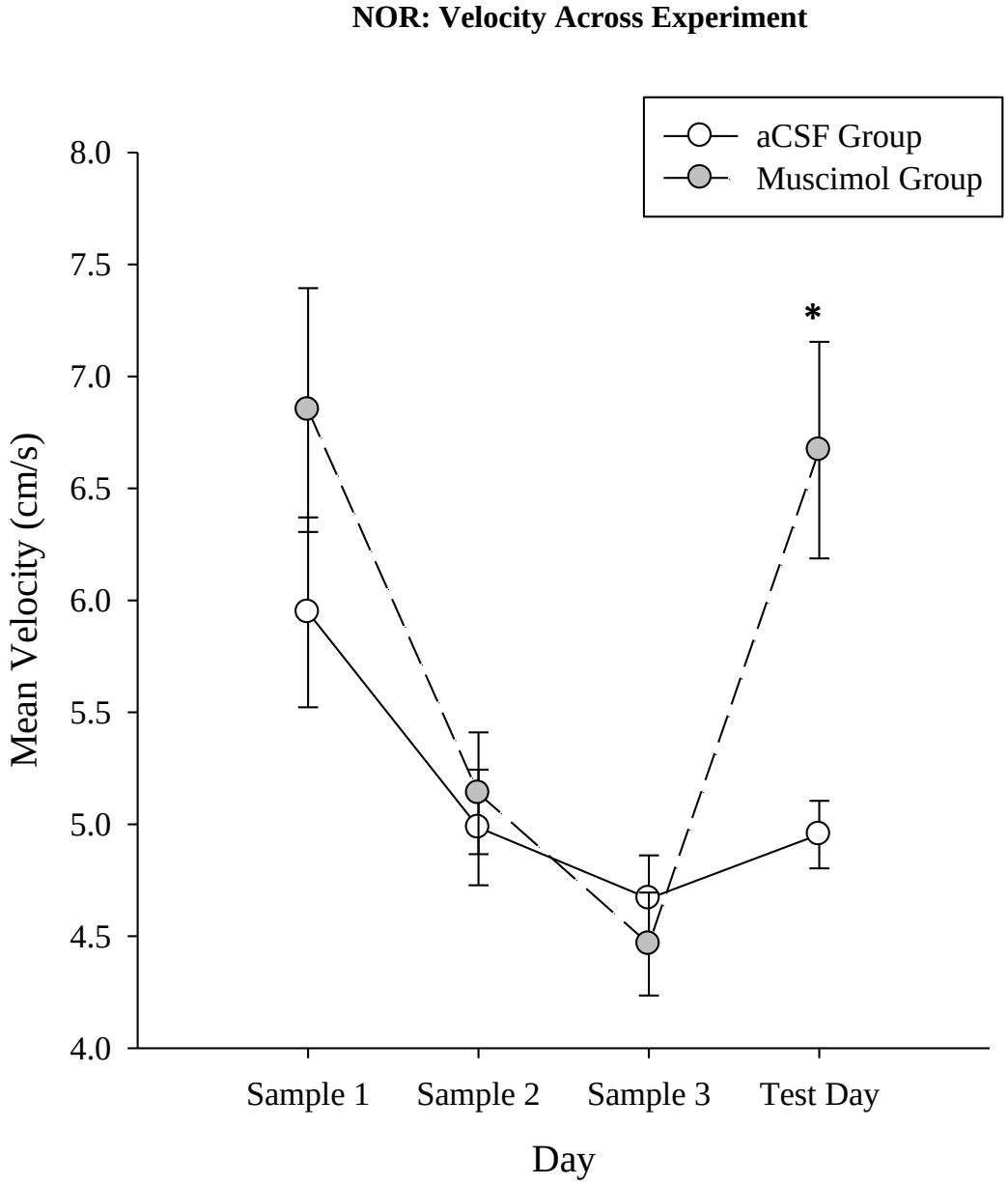


Figure 27. NOR velocities across experiment. Although the future groups did not exhibit any differences between each other in mean (± S.E.M.) velocity across sample days, they both significantly decreased their velocities from the first sample day to the third (P = 0.012). This decrease in velocity is an indication of habituation to the testing arena. On test day, the muscimol group was significantly faster than the aCSF group (t_{15} = -3.918, P = 0.001, *), but comparing velocities across all days it is evident that the muscimol group's test day velocity was not significantly different from its first day's velocity (P = 0.989). The muscimol group's regression back to its first day velocity suggests that they may have lost any prior habituation to the testing arena.

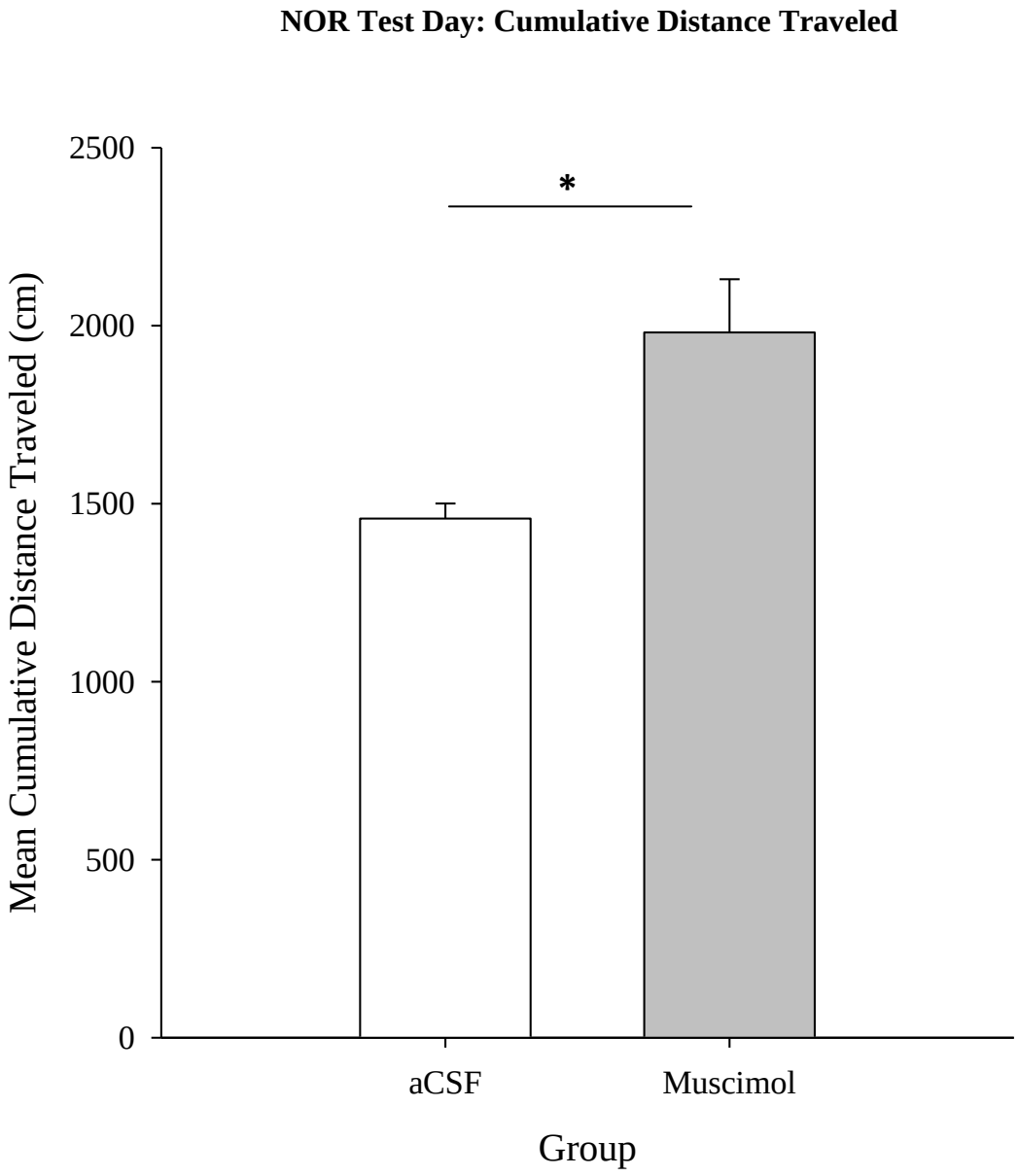


Figure 28. NOR test day cumulative distance traveled. The mean (± S.E.M.) cumulative distance traveled by mice that received muscimol microinfusions was significantly greater than that of mice that received aCSF infusions (t_{15} = -3.926, P = 0.001, *).

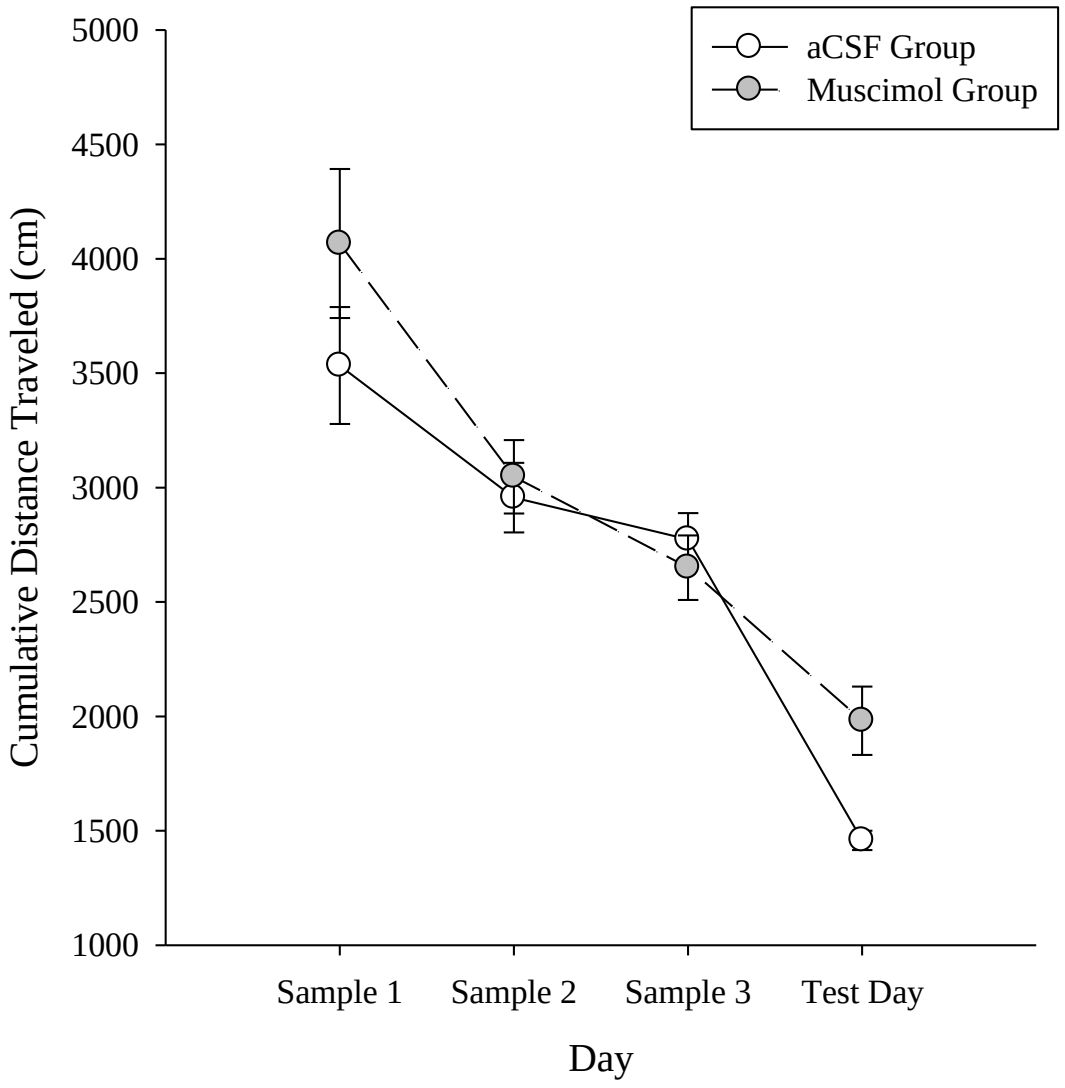


Figure 29. NOR cumulative distance traveled across experiment. Although the future groups did not exhibit any differences between each other in mean (± S.E.M.) cumulative distance traveled (CDT) across training days, the muscimol group did travel significantly more distance on the test day (figure 29). An analysis of CDT across the entire experiment reveals that CDT decreased as mice became habituated to the testing arena. On the test day, the CDT of the aCSF mice decreased again, as did the CDT of the muscimol group; however, the muscimol group's CDT did not decrease as much (perhaps because of a partial loss of the memory for the arena), resulting in the significant difference between groups' CDT on test day.

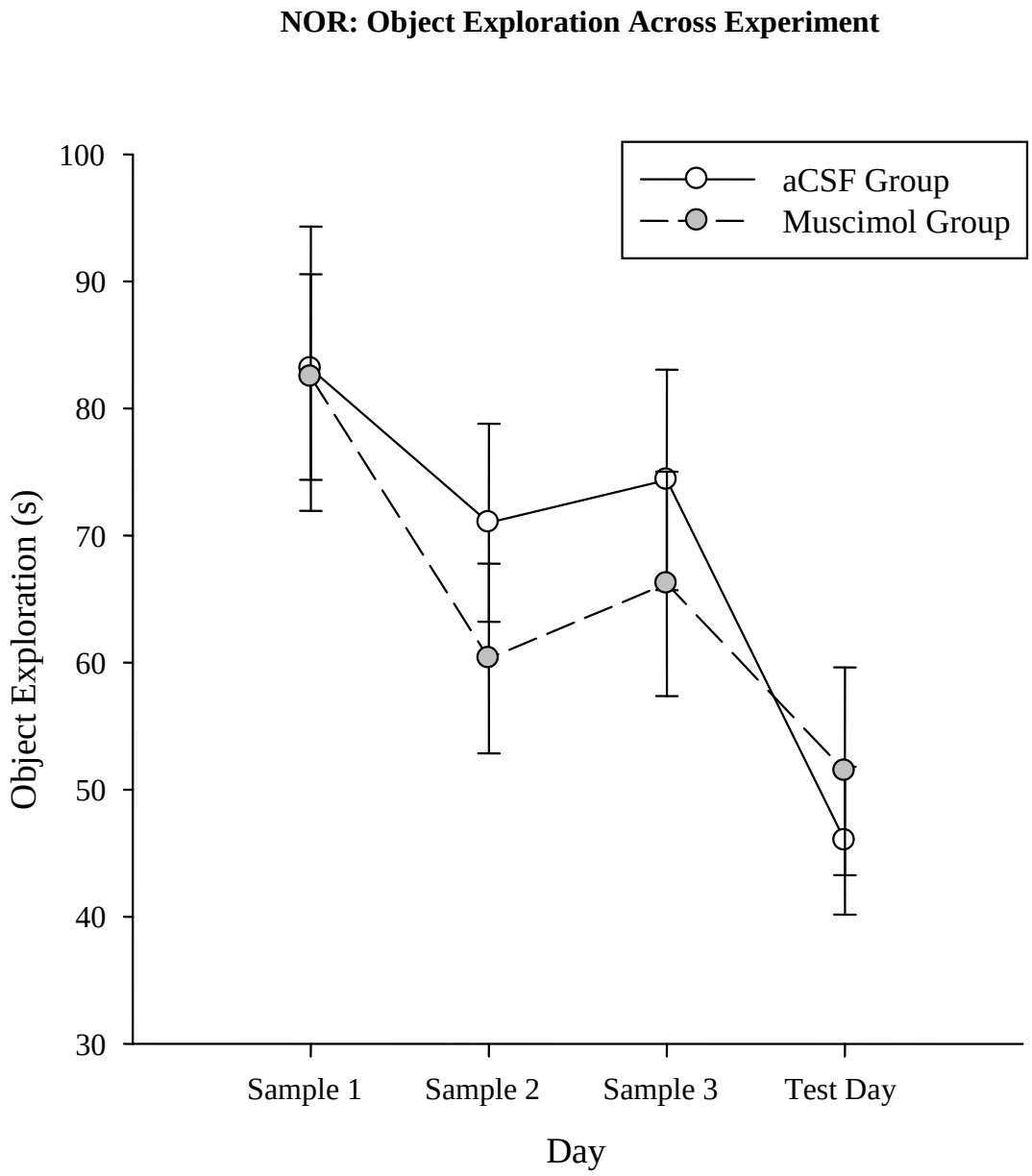


Figure 30. NOR object exploration across experiment. Mean (± S.E.M.) object exploration within groups decreased significantly across the duration of the experiment ($F_{3, 15} = 10.533$, $P < 0.001$); but the groups did not vary significantly from each other on any of the days, indicating that the groups were equal prior to test day infusions and that muscimol did not result in decreased object exploration.

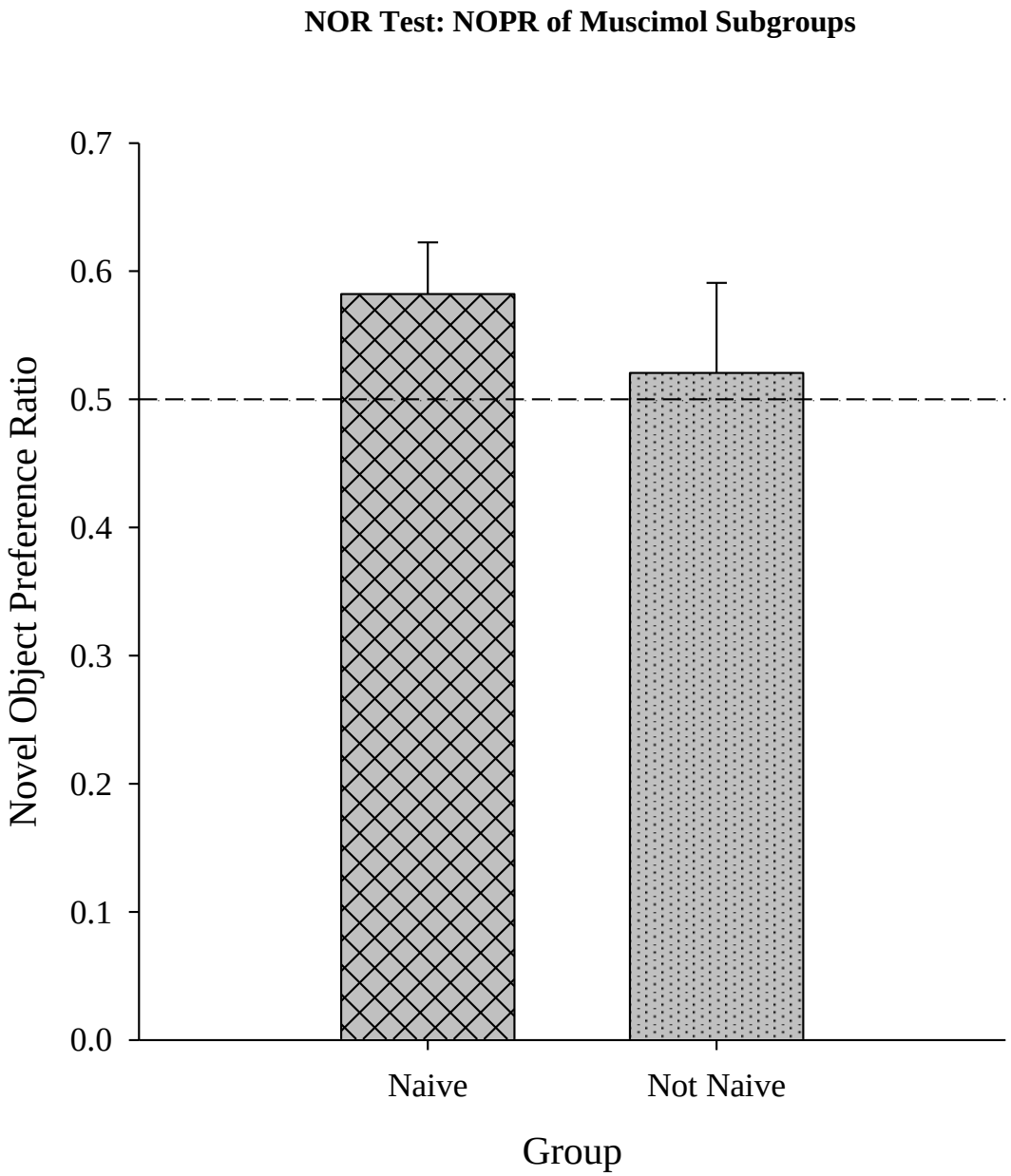


Figure 31. NOR novel object preference ratios of muscimol subgroups. The mean (± S.E.M.) NOPR of naïve mice was not significantly different than the NOPR of the non-naïve mice who had experienced the testing arena and the familiar objects 21 days prior (t_5 = 0.814, P = 0.453). Therefore, the results from the two groups were combined into one muscimol group.

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