HIPPOCAMPAL CA1 ACTIVATION DURING OBJECT MEMORY ENCODING IN THE NOVEL OBJECT RECOGNITION TASK

by

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Robert W. Stackman, Jr., Department of Psychology, and has been approved by the members of his supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Arts.

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ABSTRACT

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Transcription and translation of proteins are required for the consolidation of episodic memory. Arc, an effector immediate early gene, has been linked to synaptic plasticity following learning and memory. It is well established that the rodent hippocampus is essential for processing spatial memory, but its role in processing object memory is a point of contention. Using immunohistochemical techniques, hippocampal sections were stained for arc proteins in the CA1 region of the dorsal hippocampus in mice following two variations of the novel object recognition (NOR) task. Results

suggest mice that acquired strong object memory showed significant hippocampal activation. In mice that acquired weak object memory, hippocampal activation was not significantly different from controls. Arc expression was also examined in other hippocampal sub-regions, as well as in the perirhinal cortex. These results suggest that the mice must acquire a threshold amount of object information before the hippocampal CA1 region is engaged.

HIPPOCAMPAL CA1 ACTIVATION DURING OBJECT MEMORY ENCODING IN THE NOVEL OBJECT RECOGNITION TASK

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PART I: INTRODUCTION

1.1 The Use of Rodents to Study Human Memory Systems

Studying memory impairments following disease or trauma has allowed for the ascertainment of human memory functioning. Well studied cases, such as that of H.M. who underwent surgery to bilaterally remove the medial temporal lobes to relieve his severe symptoms of epilepsy, have provided vital evidence for the understanding of the neural substrates of memory. Although studies such as these provide us with extensive knowledge, these memory deficits cannot be replicated, manipulated, or recovered due to the inherent ethical issues that arise from conducting research on humans. Non-human studies can be carried out to further our understanding of memory functioning of the healthy brain and to characterize memory deficits after damage to specific brain regions. In order to be able to draw appropriate conclusions when using animal models of human memory, we must be sure that the specific brain regions being considered have anatomical similarities as well as similar behavioral effects between species. Rodents have proven to be a superb system to model human memory that can be examined on behavioral, cellular, and molecular levels. The suitability of rodents as animal models for human learning and memory has been the subject of much debate. Providing additional support for the similarities between the rodent and human memory system will allow future researchers to use the rodent model to answer questions regarding human learning and memory.

1.2 Multiple Memory Processes

Memory functions to store newly acquired information so that information experienced can be utilized later on. Memory involves multiple processes and can be divided into four distinct stages known as encoding, consolidation, retrieval, and reconsolidation respectively. Encoding refers to the acquisition of a memory following the initial experience. Consolidation refers to the process by which a memory is saved or stored such that it is able to be recalled at a later time. Retrieval refers to the process by which memories which have been consolidated previously are brought to consciousness and can then be utilized to modify behavior. Reconsolidation refers to the process of altering or adding to a previously consolidated memory. The process of reconsolidation can render a memory stronger, weaker, or can alter the significance of the memory.

Studies seeking to dissociate the anatomical brain regions in which specific memory processes occur have yielded extensive data with indefinite conclusions. A meta-analysis of Positron Emission Tomography (PET) studies has suggested that both encoding and retrieval occur within the hippocampus, a structure that lies within the medial temporal lobe, in human subjects. They found that both verbal and non-verbal information is encoded within the rostral portion of the hippocampus, while retrieval of that same information are processed in the caudal portion of the structure (Lepage *et al.*, 1998). Brain regions in which consolidation of memory is processed have proven to be much more difficult to identify using human subjects, however the use of animal models

has indicated that this process also occurs within the hippocampus and that the strength of the memory can be dependent on the amygdala (McGaugh, 2000).

One of the side effects precipitated by patient H.M.'s surgery was that he could no longer verbally discuss new memories for more than a few seconds. His memory deficits did not stem from the encoding of new memories and this is supported by the fact that structural Magnetic Resonance Imaging (MRI) of his brain revealed that much of the caudal portion of his hippocampus remained intact (Corkin *et al.*, 1997). H.M.'s symptoms seemed to result from the inability to consolidate or retrieve specific memories encoded after the surgery took place, which is known as anterograde amnesia.

Other cases have also demonstrated how medial temporal lobe damage causes severe memory deficits. One example is the case of R.B. who suffered an ischemic episode following heart surgery in which blood flow to the hippocampus was restricted, causing damage to the structure. He exhibited severe anterograde amnesia following the surgery that was later attributed to bilateral lesions to the CA1 region of the hippocampus (Zola-Morgan *et al.*,1986). His anterograde amnesia remained severe for the rest of his life despite not having any other notable cognitive impairment. These case studies of human patients have shed some light as to which anatomical structures are required for memory processing.

While it is clear that the medial temporal lobe is necessary for some types of learning and memory, it is evident that other types of learning and memory function independently of the hippocampus. One comprehensive review, which combined the findings of 147 case studies, concluded that bilateral hippocampal damage consistently

resulted in severe episodic memory impairments, however, they found short term memory and implicit or procedural memory abilities were preserved (Spiers *et al.,* 2001). This idea has been supported experimentally in studies using nonhuman primates. Monkeys with fornix lesions damaging hippocampal connections exhibited recognition memory impairments, while associative or familiarity memory remained intact (Gaffan 1974).

1.3 Memory Systems

Human memory can be further classified based on its duration or by its function. Short-term memory differs from long-term memory in its capacity and its duration. Long-term memories are retained longer than just a few minutes, unlike short-term memory, and after consolidation, may even last throughout the lifetime. There are two different types of long-term memory: declarative memory and non-declarative memory. Declarative memory (Figure 1 A) refers to memories that can be consciously recalled such as facts, events, and verbal knowledge. Non-declarative (Figure 1 B), or implicit, memory refers to unconscious memory such as skills like riding a bicycle. These kinds of long-term memory will be discussed in further detail in the sections that follow.

1.3.1 Non-Declarative Memory

Non-declarative memory that influences our behavior without conscious recollection is referred to as implicit memory. This consists of the knowledge or skills gained during the performance of certain activities such as riding a bicycle, as previously mentioned, or mirror drawing training. It has been shown that structures other than those found within the medial temporal lobe support implicit memory processing. Non-declarative memory is often described as various skillful behaviors such as conditioning

or skill learning. It is believed that the cerebellum along with the amygdala serve as the neural substrates for simple classical conditioning. The striatum is thought to be responsible for procedural memory processes involving simple skills. In patients such as H.M., priming and perceptual learning were found to be intact even when the medial temporal lobe was damaged, and are thought to be supported by areas of the neocortex (Squire, 2004). This view that implicit memory processing does not need to be consciously recalled has been supported by research in animals and non-human primates (Squire, 1992).

1.3.2 Declarative Memory

The term memory, as it is thought of in a traditional sense, is usually alluding to declarative memory, which is long-term memory that can be consciously recalled in a representational manner and is dependent upon functionally intact medial temporal lobes, especially the hippocampus (Eichenbaum 2000; Squire 1992). Declarative memory is subdivided into semantic and episodic memory. Semantic memory refers to the memory for ideas and concepts that are not drawn from personal experience, but are no unique to an individual and are things that are common knowledge, such as the names of colors, the sounds of letters, or other basic facts that may require effortful conscious recollection (Eichenbaum 2000). Episodic Memory is the memory of autobiographical events such as times, places, and other contextual knowledge obtained from past personal experiences that occurred at a particular time and place and requires the involvement of the frontal lobe (Squire, 2004). These episodic memories are highly dependent upon activity within the hippocampus and the surrounding cortical structures and it is this type of memory which will be the focus of this thesis.

1.4 The Hippocampal Circuit

The hippocampal formation consists of a complex circuit pathway that receives input from the neocortex via the entorhinal cortex. Fibers from the entorhinal cortex enter the hippocampal formation and synapse onto the dentate gyrus (DG) via the performant pathway. Neurons within the DG send axons forming the mossy fibers that synapse onto the CA3 layer of the hippocampus. Those neurons in CA3 then synapse onto the CA1 layer via the schaffer collaterals. The CA1 neurons then send their fibers and synapse in the subiculum and then back to the entorhinal cortex forming the classically studied tri-synaptic loop (van Strien *et al.*, 2009) (Figure 2). The formation or encoding of declarative memory is thought to be dependent on the flow of multimodal sensory information through the hippocampal formation via this tri-synaptic loop. This sensory input is received first by associative cortical regions neighboring the hippocampus such as the perirhinal cortex (PRh) and the postrhinal cortex (POR), and the information is differentially distributed throughout the parahippocampal region. The PRH sends projections to and receives input from the lateral entorhinal cortex (LEC), whereas the POR sends projections to and receives input from the medial entorhinal cortex (MEC). The MEC and the LEC are thought to receive input from two separate streams, the dorsal and the ventral streams (Figure 3). The dorsal stream processes spatial and idiothetic information, while the ventral stream processes nonspatial information consisting of olfactory, auditory, and object information. These pathways converge on the hippocampus with dorsal stream information entering through the MEC and ventral stream information entering through the LEC. These structures have been identified as critical for the processing of episodic and semantic memory.

1.5 Familiarity and Recollection

Previous research has led to a distinction between familiarity and recollection in reference to recognition (episodic) memory. Recollection involves qualitative associations, while familiarity refers to a rapidly accessible sense that an event was previously experienced (Eichenbaum *et al.*, 2007). A neuroanatomical basis was proposed by Brown and Aggleton for the recollection-familiarity distinction. They posited that recollection depends on the hippocampus, whereas familiarity depends on the adjacent perirhinal cortex (Brown & Aggleton 2001). The relational-recognition method is one strategy that was used to distinguish 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). This distinction was also observed utilizing event-related potentials (ERPs) recordings during memory tasks (Eichenbaum et al. 2007). However, later research reviewing rodent, monkey and human studies has shown evidence for a novelty signal as well as a recollection signal within the hippocampus (Squire *et al.*, 2007). Squire & Clark (2007) proposed that the previously utilized methods that separated familiarity and recollection were actually separating weak memories from strong memories. They found these same familiarity and recollection signals also occur within the perirhinal cortex. Their findings suggest that the perirhinal cortex fires robustly to visual stimuli when they are first presented, but less so when the stimuli are familiar (Squire et al., 2007).

1.6 Spatial Memory and the Hippocampus

Spatial Memory is a form of episodic memory which allows one to associate a specific event with the location in which it occurred. Rodents can quickly learn to associate a location or specific place within their environment with a reward, or to avoid it if an aversive stimulus is given, utilizing environmental cues. It has been well established that the hippocampus plays a critical role in spatial navigation and learning new environments both in humans and in rodents. The most common test used to study spatial memory in rodents is the Morris Water maze. The Morris Water maze permits the assessment of spatial memory in a circular pool filled with opaque water where rodents learn to locate a platform typically hidden beneath the surface of the water by using fixed external cues located in the environment outside of the pool. Rodents can be trained over a few days to find the platform location and can later be tested by removing the platform and measuring the time spent searching for the platform in the appropriate area of the pool where the platform used to be located. When the hippocampus is lesioned prior to training, rats are impaired in learning the location of the platform based on distal cues (Eichenbaum *et al.*,1990; Morris *et al.*, 1982).

Lesion studies, however, do not provide the most complete answer when it comes to determining the functions of specific brain regions. Asking how memory is affected after permanently destroying neurons within the hippocampal formation only tells part of the story. The permanent lesion does not make it feasible to conclude which stages of memory the hippocampus is involved in. We cannot infer whether it is in the consolidation or in the retrieval of the spatial memory for which the hippocampus is critical. The development of local infusion cannula allows for administration of drug

directly into the desired location within the brain, leaving the rest of the brain intact and functioning normally while the animal moves freely. This allows for the ability to fundamentally either silence or enhance neuronal activity within the hippocampus, thus interfering with hippocampal function during specific time periods such as during training or during the test session of the Morris Water maze task, offering empirical advantage over permanent lesion. In this way it is possible to selectively dissect hippocampal contributions during the consolidation and retrieval stages of memory respectively. When a selective AMPA antagonist is chronically infused into the CA1 region of dorsal hippocampus during training, rats show impaired acquisition as measured by significantly longer escape latencies (Riedel et al. 1999). This evidence suggests that the hippocampus is essential for the consolidation of new spatial memories. When the hippocampus of a successfully trained rat is temporarily inactivated before the test session in the water maze, rats exhibit spatially localized search behavior in the incorrect location of the pool (Riedel et al. 1999). These results indicate that a fully functioning hippocampus is essential for retrieving information about a particular location, while the searching strategies that the animal employs remain unimpaired and are therefore likely dependent on brain regions other than the hippocampus.

One explanation for the inability of an animal to appropriately navigate their spatial environment due to hippocampal dysfunction comes from the discovery of place cells by O'Keefe and Dostrovsky in 1971. Place cells are pyramidal neurons that reside within the hippocampal formation and fire at high frequency corresponding to the animal's location within its spatial environment. This location is referred to as the cell's "place field." These cells are silent when the animal is out of the place field but generally

remain stable (fire at same location at similar Hz) when the animal returns to that same area (O'Keefe & Dostrovsky 1971). The activity of these cells is observed via extracellular recordings which are carried out by surgically placing tetrodes into the hippocampus of freely moving animals, enabling the recording of action potentials from nearby cell bodies. These findings offer support for the link between the hippocampus and spatial memory function as it is likely that when the hippocampus is temporarily inactivated, these place cells are also inactive resulting in spatial processing deficits, thus precipitating navigational impairments.

It has been shown that place cells in the hippocampus are influenced by grid cells which reside in the medial entorhinal cortex (Brun *et al.*, 2008; Fyhn *et al.*, 2007) and also by head direction cells found in the anterior thalamus (Yoganarasimha & Knierim 2005; Calton et al. 2003). Grid cells have firing properties that resemble those of place cells except that grid cells fire at multiple locations laid out in a hexagonal pattern across a familiar environment. Grid cells make synapses with place cells residing in the CA3 and CA1 regions of the hippocampus, and have been shown to disrupt hippocampal place cell firing when the entorhinal cortex is impaired (Brun et al. 2008). When an animal is placed in a novel environment, place cell remapping occurs accompanied by a coordination shift in grid cells. Head direction cells, on the other hand, are influenced by a combination of proximal and distal environmental cues and have been shown to mediate the control exerted by distal landmarks on place field representations. These cells fire in response to the animal's head direction along its horizontal plane and are independent of the animal's location in space. Functioning of head direction cells is highly influenced by input from the vestibular system (Jacob *et al.*, 2014), and they have

been shown to influence place cell remapping in a familiar environment when the animal is repeatedly disoriented (Knierim *et al.*, 1995). These three types of cells are the major contributors to an animal's perception and processing of space which function to create a representation of direction and location within an environment (Brun et al. 2008).

1.7 Non-Spatial Memory and the Hippocampus

It is important that before utilizing rodent models to study functions of human memory, that we determine if the rodent hippocampus plays an important role in non-spatial memory similar to that of the human hippocampus (Cave & Squire 1991). The Novel Object Recognition task (NOR) is a test of episodic object memory (Figure 4). The NOR task is carried out using a familiar environment that has minimal environmental cues, which contains objects that the animal is free to explore for a short amount of time. Animals are first exposed to two arena sessions that serve to familiarize the arena and eliminate any interference of novel context information processing. The first object session, referred to as the sample session, contains two identical objects in separate locations within the arena. The subject animal will typically explore both objects similarly, indicating that the two objects are novel to the animal. The following object session, referred to as the test session, occurs in the same environment as the sample session containing one of the previously exposed objects in one position of the arena, and in the other position is a novel object that the animal has never been exposed to (positioning of the objects within the arenas is consistent across trials). The animal freely explores these objects and the time that the animal spends observing each object is observed and carefully recorded. This task takes advantage of the natural curiosity of mice to explore novel objects in their environment more than objects they are familiar

with (Ennaceur & Meliani 1992). Rodents that fully encode a memory for the objects that they were exposed to in the sample session should retrieve that memory during the test session. As a result of remembering the sample objects, the animal should spend a greater amount of time exploring the object that was not present during the sample session. Rodents that do not fully encode the memory of the sample objects should not have the ability to retrieve the memory for those objects, and should thus spend an equal amount of time exploring both test session objects.

The novel object recognition task is widely used to test the recollection of objects and two brain regions have been identified as playing a crucial role in this form of memory. Some researchers have identified the perirhinal cortex as being responsible for object recognition in primates and rodents (Buffalo et al. 1999; Winters & Bussey 2005), but others argue that the hippocampus is the critical brain region for object memory (Broadbent *et al.*, 2010; Clark *et al.*, 2000; Clarke *et al.*, 2010; Cohen *et al.*, 2013; Fortin *et al.*, 2014). In these studies, testing on both brain areas has been carried out similarly but conflicting conclusions have been found. It is likely that both the hippocampus and the perirhinal cortex play an important role in the encoding and retrieval of memories for objects, but it is possible that these contradicting results are due to differences in experimental protocol. In a study with rats in which the perirhinal cortex (PER) was lesioned (Winters & Bussey 2005), animals were tested in a Y-maze NOR task. PER lesioned rats demonstrated equal preference for the novel and familiar objects, suggesting that the perirhinal cortex encodes and/or consolidates object memory. Other studies have found that the perirhinal cortex ploys an important role in visual perception of objects, which may provide an explanation for the deficits seen during object recognition. It is

possible that in the Winters et al. study, object memory may still have been intact, and the deficit in the NOR task may have been produced by a perception impairment instead of a memory impairment. Before these results can be properly interpreted, it is important to be able to rule out the possibility of perceptual impairment interfering with the cued retrieval of object memory in the NOR task. Extracellular activity was recorded in the perirhinal cortex in rats as they ran around a circular track either in the presence or in the absence of 3D objects (Burke et al. 2012). They found object-related activity in the perirhinal cortex; however, it was not specific to an object's identity and firing was not modulated by experience. This evidence supports the idea that the perirhinal cortex is important for object perception, but that it is likely not responsible for object memory.

A considerable amount of research has been done looking at object memory processing in the hippocampus. Studies have shown that when the CA1 region of the dorsal hippocampus is temporarily inactivated in rodents during the various stages of NOR, memory of objects is impaired, producing deficits in both the encoding and retrieval of object memory. These findings suggest that object memory processing occurs within the hippocampus (Clark et al. 2000; Cohen et al. 2013). One of the main criticisms of this research argues that the reason object memory impairments are observed in hippocampal inactivated rodents in the NOR task is because the hippocampus is actually encoding object-in-place memory as opposed to purely memory of the object. It has also been reported that after temporary inactivation of CA1 via infusions of muscimol in the dorsal hippocampus after only one habituation session resulted in enhanced novel object recognition memory (Oliveira *et al.*, 2010). The same study also found that when the same experiment was repeated with more habituation sessions to familiarize the mice to

the context, this enhanced novel object recognition memory was eliminated. These conflicting results may be attributed to familiarization to the testing environment prior to testing. The increased preference of hippocampal inactivated mice for the novel object during the test session was only observed when the mice received one habituation session within the testing context. Familiarization with the environment, or lack thereof, could be a confounding factor that makes it more difficult to explore true object memory. It is also important to note one key issue with this study, which was that object exploration on the sample and test sessions varied greatly between the two experiments.

It can be difficult to differentiate objects from environment; objects contribute to our surroundings and may serve as landmarks. Since no object can be encountered in the absence of context, this problem can be difficult to address. It is important that the testing arena is absent of cues in order to limit the spatial information that is available to the animal. Studies have shown that changing the location of the familiar object in a well-known arena results in increased exploration (Mumby *et al.*, 2002) suggesting that the rat recognizes something has changed about the object, but a novel object in a novel location results in even more extensive exploration. It has been suggested that this object-in-place or object-in-context memory is hippocampal dependent due to the increased spatial and contextual nature of the task. Cohen *et al*. (2013) tested object memory that was independent of context by giving mice the same sample session objects for 10 min on 3 consecutive days with a different environment each day. They found that when the hippocampus is intact, mice are equally successful at retrieving the object memory in an environment that they are familiar with, as they are in a context they have never before encountered. However, when the CA1 region of dorsal hippocampus is temporarily

inactivated via intracranial injection of muscimol before the test session in a novel environment, the mice cannot perform the task (Cohen et al. 2013). This result suggests that although the hippocampus is processing spatial and object information, the location of the object can be processed independently of the object information itself.

In a study done by Cohen *et al.* (2013), NOR was run with a strict criterion placed on the 10 min sample session; a minimum 30 seconds of exploration of each object or 38 seconds of one of the objects; mice who did not acquire this criterion were excluded from analysis in the 5 min test session (Cohen et al. 2013). Using an object exploration criterion such as this reduces the possibility that mice do not obtain enough information about the sample objects to fully encode the memory and controls for the sample object experience across mice. Surprisingly, there are very few published studies that account for this and impose a sample session object exploration criterion. Instead, the majority of these publications opt for a set time of exploration for the sample session (e.g. 2 min). requiring mice to explore the sample objects for 30 seconds each leads to a stronger memory for the objects, and thus a strong preference for the novel object during the test session when given 24 h later (Cohen *et al.*, 2013; Hammond *et al.*, 2004). It is possible that this difference of exploration during the sample session could account for the conflicting results presented by Oliveira *et al.* (2010), which reported and average of 22.9 sec of sample object exploration.

Lesion studies have also presented conflicting results regarding the role of the rodent hippocampus in object memory. It was suggested that the size of the hippocampal lesion contributed to impairments in object recognition. One study described significant impairments in the NOR task using a protocol that had previously been shown to be

affected by hippocampal lesion when the entire hippocampus was lesioned in rats, however, partial lesions spared the object memory (Ainge *et al.* 2006). This phenomenon does not correlate with temporary inactivation results; significant impairments are seen when just 1% of hippocampal volume is inactivated (Cohen *et al.*, 2013; Hunsaker *et al.*, 2007). A review done by Squire *et al.* 2007 reports that about half of the studies aiming to deduce whether the rodent hippocampal lesions affect novel object recognition memory show no impairments during the task. The other half that demonstrated significant impairments did so when the interval between the sample session and the test session exceeded 10 min (Mumby *et al.* 2002; Clark *et al.* 2000). Squire *et al.* proposed that the strength of the memory can determine which medial temporal lobe structures are processing the information. They found that strong memories were associated with increased activity within the hippocampus, while weak memories did not require the hippocampus, but may be dependent on neighboring cortical areas, of which they named the perirhinal cortex as the prime suspect for object memory. They define strong memory as being based on recollection, while weak memory is defined along the lines of recognition such that the original experience could not be remembered unless the previously experienced stimuli were present, similar to findings of human fMRI data (Squire *et al.*, 2007).

It seems that the delay period between the sample session and the test session of the NOR task plays an important role in how we interpret which structures support object memory. Findings from both lesion studies and temporary inactivation studies have concluded that object memory is not hippocampal dependent within 10 min of encoding after the sample session. When the dorsal hippocampus is inactivated by injecting

lidocaine before the sample session and tested 5 min after, no impairments are seen (preference for the novel object). When tested 24 hours after receiving the pre-sample lidocaine injection, mice perform at chance and show no preference for the novel object (Hammond et al. 2004). Similar effects were seen in lesion studies. When 75% of the hippocampus was lesioned, object recognition was spared when the rats were given the test session up to 1 min following the sample session. If this delay exceeded 10 min, however, impairments were reported (Clark et al. 2000). Moreover, hippocampal lesioned rats did not demonstrate any impairments when tested with up to 3 min delay intervals between sample and test sessions, although the rats were impaired in discriminating between object locations after they had been displaced within the same delay intervals (Mumby et al. 2002). These findings suggest that it may require some time to consolidate object memory to form a strong, hippocampal dependent memory, while spatial information is processed by the hippocampus much more quickly.

Hippocampal object memory may be relocated to other cortical areas once it has been fully consolidated. Rats who received hippocampal lesions 1 day, 4 weeks, or 8 weeks after receiving 12 sample sessions, demonstrated differential effects on object recognition when the test session was given 2 weeks after the surgery. The 1 day and 4 week consolidation interval rats showed object recognition impairments when compared to their respective control group, however, the 8 week consolidation interval rats did not demonstrate any impairments in recognition of the sample objects (Broadbent et al. 2010). To confirm that the hippocampus was essential for object memory, the rats were also given an additional NOR protocol using unique object pairs and a 3 hour delay between sample and test sessions resulting in consistent and moderate memory

impairment in all lesion conditions. These findings suggest that the hippocampus may only be necessary for these memories during a critical period before the memory becomes permanently stored in a distributed network outside of the structure.

It is still largely unknown how the perirhinal cortex in conjunction with the hippocampus jointly encodes memories for objects. There is one unifying theory that has been proposed by Cohen & Stackman (2014), which proposes a mechanism by which object recognition may be supported by both the perirhinal cortex and the hippocampus. Their review found a key difference in the methods between researchers who found that object recognition memory tested in the NOR task was solely perirhinal dependent and those that found it to be hippocampal dependent; that difference being the amount of time animals explored the sample objects. Sample session object exploration of 30 seconds or more on each object appears to be a condition that is required to engage the hippocampus or to shift control over the memory from perirhinal to hippocampus. Their proposed Bucket Theory postulates that prior to the start of the NOR sample session, both the perirhinal cortex and the hippocampus lack sample object information, and as object exploration commences, the perirhinal cortex accumulates information and it remains in the perirhinal cortex until a minimum amount of object exploration is achieved (~30 s on both or 38 s on one). Once this critical threshold has been reached, the information begins to transfer over to the hippocampus (Cohen & Stackman, 2014). This new theory proposes a viable mechanism to support object recognition memory and provides a possible explanation that may bridge the gap that exists in the NOR research.

1.7.1 Object Related Neuronal Activity

Recent studies have looked at how place cells react to objects placed within a familiarized environment. Extracellular electrophysiological recordings done in the dorsal CA1 region of the hippocampus in rats did not demonstrate significant place field remapping between recording sessions. Place fields residing proximal to the location of an object in the environment remained stable whether the object was present or missing and even when the object was replaced by a novel one. There were some cells, however, showing firing patterns that were sensitive to the object in a specific location (Burke *et al.*, 2011). These findings indicate that there may be cells within the hippocampus, specifically within CA1, that process object information that is independent from object location alone. It has also been suggested that the presence of objects affects the firing properties of place cells. One study recorded from dorsal CA1 and CA3 of the rat hippocampus found differential activity due to different objects that were placed in the same location at different times indicating that these objects were "points of interest" on the place cell map (Manns & Eichenbaum 2009). More recent work recording from the same locations found a new type of place cell know as landmark-vector cells, which fired at a specific direction and distance from an object within the environment (Deshmukh & Knierim 2013). These landmark-vector cells did, however, retain their firing properties when the object was removed or relocated from the arena, but were also able to adopt new firing fields following the new location of the object while retaining the properties of the old location as well. These results strengthen the argument that the rodent hippocampus process object information and that object memory is retained within it.

1.8 Immediate Early Gene Staining

Classical studies have demonstrated that protein synthesis plays an important role in long-term memory (Abraham & Williams, 2008; Fifková *et al.*, 1982; Frey *et al.*, 1988; Krug *et al.*, 1984; Otani *et al.*, 1989; Staubli *et al.*, 1988) as well as rapid mRNA synthesis (Flexner *et al.*, 1963), and one of the key mechanisms by which this is accomplished, is the transcription of immediate early genes, which occurs quickly after a neuron has been active. Immediate early genes are widely believed to play a critical role in transformation of activity in neural circuits into long-term memories in the brain (Jones et al. 2001). Immunohistochemichal staining techniques targeting these proteins have allowed for the mapping of specific functions onto sub-regions within the hippocampus, such as the encoding of context-specific information (Kubik *et al.*, 2007) and the discrimination between novel and familiar objects (Albasser *et al.*, 2010) in CA1 and CA3. Inhibiting proteins translated from immediate early gene RNA hinders the persistence of inhibitory avoidance long term storage (Katche et al. 2010). It has been suggested that the perirhinal cortex produces a novelty signal that affects hippocampal processing, with greater familiarity engaging the perirhinal cortex to a lesser degree (Fernández & Tendolkar 2006). This increase in perirhinal processing triggers a response in the hippocampus following the onset of a stimulus, which has been shown to lead to a stronger memory that is more likely to be remembered later. This idea has been supported by c-fos (an immediate-early gene) protein staining following the exploration of novel and familiar objects; fewer c-fos positive cells were found in the perirhinal cortex of rats who explored familiar objects than rats who explored novel objects (Kinnavane *et al.*, 2014). These findings seemingly contradict the findings previously mentioned that found that hippocampal lesions do not impair memory for familiar objects in the NOR task. The

goal of this thesis is to clarify the role of hippocampal CA1 in object recognition memory by staining for the immediate early gene Arc. Arc is an effector gene that plays a crucial role in synaptic plasticity processes by directly influencing cellular structure and functions. Arc is enriched in neuronal dendrites following long term potentiation inducing stimulations, suggesting a link between Arc and synaptic as well as whole cell plasticity (Sauvage *et al.*, 2013). It has also been shown that Arc knockout mice fail to form long-lasting memories for implicit and explicit learning tasks (Plath et al. 2006). Presumably, when new memories are being formed, the Arc gene is transcribed in cells that are actively representing that memory, and Arc proteins are produced to mobilize the formation of new connections between cells as well as the strengthening of particular connections involved in the memory.

1.9 Current Study: Purpose and Hypothesis

Considering the studies mentioned previously, additional evidence is needed to demonstrate that object memory in the rodent brain is hippocampal dependent. The present study was designed to demonstrate the nature of hippocampal activation during the novel object recognition task using immunohistochemical techniques. It asks the question of whether hippocampal involvement in object memory is based on a minimum threshold of information obtained about the object. If staining against Arc Proteins in the hippocampus during two modified versions of the NOR protocol reveals that there is greater hippocampal activity during a strong memory protocol than in a weak memory protocol and that activation during a weak memory protocol is the same as baseline activity, this could be interpreted as evidence that the hippocampus processes object memory in a strength dependent manner.

As described earlier, many argue that the NOR task requires the rodent to encode a memory of the object-in-context in order for the task to be hippocampal dependent. This study incorporates ample arena familiarization or habituation before object testing occurs in order to reduce novel context processing within the hippocampus to a minimum. The novel object recognition task was carried out utilizing two different object exploration criteria for each respective protocol. Determining that the rodent hippocampus is engaged during novel object recognition due to processing of the object memory, and not only due to spatial processing of the testing arena provides further support for the importance of the rodent hippocampus for object memory. Finding that the strong memory sample mice show greater hippocampal activation than the strong memory arena habituation animals will strengthen the case that the memory for the sample objects is dependent on the hippocampus. Finding that the weak memory sample animals show no difference from the arena habituation animals will support the idea that the hippocampal dependence of object memory is based on the strength of the memory that is encoded. Finding that the perirhinal cortex is more active in the weak memory sample mice than in the strong memory sample mice will lend support to the idea that weak object memories are dependent on the perirhinal cortex instead of the hippocampus. It is important to define how the rodent hippocampus processes object information in the novel object recognition task in order to be able to accurately compare the rodent hippocampus as an appropriate model of the human hippocampus.

Protocol 1: *Strong Memory NOR.* In order to assess hippocampal activation during object memory consolidation, the Novel Object Recognition paradigm will be carried out on three cohorts of mice, utilizing an object acquisition criterion that has been

shown to yield an object memory that is hippocampal dependent (30/38 s). Mice will receive two habituation sessions in the empty arena, followed by a sample session with two identical objects, and then by a test session containing one familiar object from the sample session and one novel object the animal has never been exposed to. Control mice will receive a third arena habituation session instead of the sample session, in which no objects will be present. Mice will be euthanized 90 minutes after each respective session and brain tissue will be harvested and processed.

Protocol 2: *Weak Memory NOR.* In order to help construe the role of the hippocampus in object memory, the Novel Object Recognition task will be carried out utilizing an object acquisition criterion in which it has been shown to yield a memory for the object that is not dependent on the hippocampus (10/13 s). Mice will receive two habituation sessions in the empty arena, followed by a sample session with two identical objects, and then by a test session containing one familiar object from the sample session and one novel object the animal has never been exposed to. Control mice will receive a third arena habituation session instead of the sample session, in which no objects will be present. Mice will be euthanized 90 minutes after each respective session and brain tissue will be harvested and processed.

PART II: MATERIALS AND METHODS

2.1 Subjects

Subjects used for the novel object recognition task were male 7-10 week old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME). Thirty-two mice were housed in groups of four in polycarbonate home cages in a vivarium which was humidity and temperature controlled. Room temperature and humidity were maintained at 22 ± 4º C and 50 ± 5%. A 12-hour light/dark cycle was maintained beginning at 7:00 AM. All experimental procedures were conducted during the light period. Home cages were housed on a ventilated rack and mice received *ad libidum* access to food and water throughout the experimental period. Prior to testing, mice were given one week of vivarium acclimatization. Animal use procedures were carried out in accordance with the guidelines required by the National Institute of Health Guide for the Care and Use of Laboratory Animals. The Florida Atlantic University's Institutional Animal Care and Use Committee obtained approval for all procedures prior to experiments.

2.2 Immunohistochemistry

At the conclusion of behavioral testing, mice were deeply anesthetized with sodium pentobarbitol. Each mouse was transcardially perfused with phosphate-buffered saline, followed by 4% paraformaldehyde and brains were dissected and preserved in 4% paraformaldehyde. Brain slice sectioning was conducted using a cryostat to obtain sections 30 µm thick. Sections were then stained using a standard immunohistochemical

technique targeting the immediate early gene Arc (Figure 7). Sections were first quenched by incubation in hydrogen peroxide. Endogenous peroxidase was then blocked with a triton and normal goat serum solution in phosphate buffered saline. Sections were next incubated in phosphate buffered saline containing Arc rabbit polyclonal antibody, and normal goat serum. Sections were then washed with phosphate buffered saline and incubated for Arc in biotinylated goat anti-rabbit secondary antibody and normal goat serum. Sections were then washed and processed with avidin-biotinylated peroxidase enzyme complex in phosphate buffered saline. The reaction was then visualized using diaminobenzidine and finally stopped by washing in phosphate buffered saline. Sections were then mounted on gelatin-coated slides, counter stained using cresyl violet, dehydrated through a graded series of alcohols and coverslipped. Every third section (30μm) was taken during slicing such that each section was 90 μm apart from the previous. To quantify arc-positive neurons in CA1 of the hippocampus, images were taken from coronal sections at the septal, intermediate, and temporal levels. Two bilateral images were matched from each respective level so that six sections were counted for each animal.

2.3 Cell Counts

Once sections were coverslipped, they were visualized using a compound light microscope. Images were viewed on a Nikon Eclipse 55i microscope and photographed using a Nikon DS-Fi1 camera at 100x magnification. Estimates of Arc-positive cells were made by the primary experimenter, in the region of interest, hippocampal CA1 as well as in other secondary regions of interest including hippocampal CA3, dentate gyrus, and perirhinal cortex. The counter was blind to experimental condition by randomizing

sections and reorganizing them after counts were made. Counts of labelled nuclei in the region of interest were determined by counting those nuclei stained above an individually determined threshold of intensity greater than background levels. A portion of the sections were selected at random to be recounted and compared to the original counts. This recount confirmed that the experimenter was consistent and precise in the counts made for each respective section.

PART III: NOVEL OBJECT RECOGNITION

3.1 Novel Object Recognition Materials

Two 37.5 x 37.5 x 50 cm high acrylonitrile butadiene styrene (ABS) arenas were located in the testing room located near the vivarium. Mouse behavior was captured with Ethovision XT tracking software (Noldus Information Technology, Leesburg, VA) via a video camera suspended above the arena floor directly above the border walls separating the two arenas. The live feed was displayed on a computer screen which tracked the movement and object exploration of the mice. Sample and test session objects were either stainless steel cabinet leveling feet (Figure 5 A), each attached to a Plexiglas base, 4.2 cm in diameter and 6.0 cm tall, or plastic toy gorillas (Figure 5 B) attached to a Plexiglas base.

3.2 Novel Object Recognition: Weak vs. Strong Memory

Mice began testing at 8 weeks old, after one week of vivarium acclimatization. The novel object recognition task apparatus consists of two open-top, high-walled square arenas made of white ABS. During day 1 and 2, each mouse was habituated to one of the arenas for a 10 min empty arena habituation session (AH1 & AH2). Then, on day 3, each mouse received either a sample session or a third arena habituation session in the familiar arena. During the sample session, each mouse was returned to the familiar arena that contained two identical novel objects positioned on the arena floor in the NW and SE corners. In the Weak Memory Protocol, each mouse was removed from the arena upon accumulating 10 sec of exploration of each object, 13 sec on either object, or if 10 min

had elapsed. In the Strong Memory Protocol, the mice were removed after they acquired 30 sec of exploration of each object, 38 sec on either object, or if 10 min has elapsed. This sample object exploration criterion was imposed to ensure that all mice were matched for sample session performance. The first cohort of mice was euthanized and perfused after completing the session. A second cohort of control mice received a third Arena habituation (AH3) session instead of the sample session (see Figure 6), in which they were yoked to a sample mouse such that they spent the same amount of time in the AH3 session as their respective matched sample session mouse spent to reach acquisition criterion on the sample objects. A third small cohort of mice received a sample session followed by a test session. During the test session, presented 24 h later, the familiar arena contained one familiar and one novel object. The mouse was allowed to freely explore and was then removed from the arena after 5 min (Figure 6). Once removed from the testing room, all mice were placed in a quiet sound proof room for 90 min after which they were euthanized. The objects, arena floor, walls and insert were cleaned with 10% ethanol after each session. Object exploration was confirmed off-line from the digital video files by experimenters that were blind to the treatment condition of the mice. Object memory was inferred from the discrimination ratio – calculated for each mouse by subtracting the time spent exploring the familiar object from the time spent exploring the novel object and dividing the result by the total time spent exploring both objects during test session. Discrimination ratio scores range from -1 to 1, with positive scores indicating novel object preference, while a ratio = 0 indicating chance performance or a lack of preference for one object over another.

3.3 Subject Clarification

Thirty-two mice were used to run the weak memory and strong memory NOR testing. Eight mice were euthanized for each respective analysis group including strong memory arena habituation 3, strong memory sample session, weak memory arena habituation 3, and weak memory sample session 90 min after the conclusion of each particular experimental session. Brains were extracted and tissue was processed and stained according to the immunohistochemistry methods. Due to over-expression of the secondary antibody during the staining process, 2 animals were excluded from analysis from the strong memory sample group (n=7) and from the weak memory arena habituation 3 group (n=7).

3.4 Statistical Analyses

Behavioral performance in the NOR task is assessed utilizing a discrimination ratio for the performance of the mice during the test session. The discrimination ratio is a formula that takes the amount of time spent exploring the novel object, minus the amount of time spent exploring the familiar object, divided by the total exploration time ($T_{Novel\ object} - T_{Familiar\ object} / T_{Novel\ object} + T_{Familiar\ object}$). Brain sections were visualized and the experimenter who was blind to condition quantified arc positive cell counts within the CA1 layer of the hippocampus as well as in the CA3, dentate gyrus, and perirhinal cortex regions (Figure 7A & 7B). The counts were averaged for each mouse, 6 sections per mouse taken from three relative areas of the dorsal hippocampus (dorsal, medial, ventral) (Figure 8). Mice in the strong memory sample group were compared to the strong memory arena habituation 3 group. The weak memory sample group was also compared to the weak memory arena habituation 3 group. Finally, mice in the weak memory arena

habituation 3 group were compared to mice in the strong memory arena habituation 3 group as a control parameter. Each group comparison was done using an Independent-samples t-test respectively with the confidence level set at 95%.

PART IV: RESULTS

4.1 Discrimination Ratio Comparison

Previous studies exploring the nature of explicit memory functioning within the medial temporal lobe has suggested that instead of thinking of the hippocampus as being responsible for recollection memory, while the perirhinal cortex supports familiarity memory without the involvement of the hippocampus, it may be more appropriate to think of memory in terms of the strength of the memory. Findings from this research suggest that memories that are strong are supported by hippocampal functioning, while memories that are weak, especially object memory, are supported by perirhinal functioning independent of the hippocampus (Squire *et al.*, 2007). In light of this concept of hippocampal dependent memory, the present study sought to employ behavioral tasks that could produce both strong and weak object memories in the strong memory and weak memory protocols respectively. In the Novel Object Recognition task, the strength of the memory for the familiar object during the test session can be quantified by measuring the preference for the novel object and is calculated as the discrimination ratio. The mean discrimination ratio for the strong memory test session mice (n=8) was significantly greater than the mean discrimination ratio of the weak memory mice (n=10) ($t(16) = -2.17$; $P < 0.05$) as was revealed by an independent samples t-test after performing square root data transformation to regain a normal distribution (Figure 9). This stronger preference for the novel object in the strong memory test mice ($DR = 0.37$),

as compared to the weak memory test mice (*DR* = 0.18), establishes that the two protocols have successfully produced strong and weak object memories respectively.

4.2 Strong Memory AH3 vs. Strong Memory Sample

Results from hippocampal lesion studies as well as studies that temporarily inactivated the hippocampus in rodents performing the NOR task have shown object memory impairments in the task demonstrated by a lack of preference to explore the novel object (Broadbent *et al.* 2010; Clark et al. 2000; Cohen *et al.* 2013; Fortin *et al.* 2014). There are many studies, however, that have found conflicting results using similar techniques. This thesis aims to demonstrate that the hippocampus does in fact process strong object memory. Hippocampal activity, as quantified by arc protein staining within the CA1 region, was significantly greater in the strong memory sample group (n=7) than the strong memory AH3 group (n=8) ($t(15) = -2.71$; $P < 0.05$) (Figure 10 A). It is likely that this increased hippocampal activation was due to the exploration of the sample objects which were not present in the arena for the AH3 group. Each individual mouse in the AH3 group was yoked to a randomly selected counterpart in the sample group in terms of time spent in the testing arena so it cannot be argued that the increased hippocampal activity in the sample group was due to a greater amount of processing due to different amounts of time spent in the NOR arena. The mean distance moved by the mice in the Strong AH3 session (2,665.63 cm) was not significantly different than the distanced moved by Strong Sample session mice (2928.59 cm) ($t(14) = 0.60$, $P > .05$) (Figure 9B). This suggests that the Sample session mice did not perceive the context to be novel when the Sample objects are present; if they had, one would expect to see greater amounts of exploration in this group, and thus greater distance moved overall. If the

hippocampal activation seen here was due to contextual processing, these two groups should not be different in terms of CA1 activation.

4.3 Weak Memory AH3 vs. Weak Memory Sample

It has been suggested by previous research that the hippocampus is differentially involved in object memory based on the strength of the memory, with other surrounding cortical areas supporting the memory if it is weaker (Squire *et al.*, 2007). Some results reported that lesions to the perirhinal cortex precipitated object memory impairment in the NOR task while lesions to the hippocampus did not produce such deficits (Buffalo et al. 1999; Winters & Bussey 2005). One possible explanation for these findings is that the versions of the NOR task used by these studies did not allow for the formation of a strong memory for the sample objects (Cohen & Stackman, 2014). In the weak memory NOR protocol done in this study, hippocampal CA1 activation in the weak arena habituation 3 group (n=7) was not significantly different from CA1 activation in the weak sample group (n=8) ($t(13) = -0.27$; $P > 0.05$) (Figure 10 B). As in the Strong memory protocol, mice in the weak memory AH3 group were yoked to animals in the weak sample group to control for the amount of time spent in the empty arena. The fact that the sample group did not have higher activation than the AH3 group may be due to the limited duration that the mice were allowed to explore the objects in this protocol. The discrimination ratio data indicate that 10 s on each object is not enough to encode a strong memory that would be dependent on the hippocampus.

4.4 Strong AH3 vs. Weak AH3

It is important to ensure that the control AH3 groups used for comparison for the weak and strong memory protocols are comparable themselves. A third independent

samples t-test analysis was done between the strong AH3 group (n=8) and the weak AH3 group (n=8) which concluded that hippocampal CA1 activation was not significantly different between the two groups ($t(13) = -1.84$; $P > 0.05$) (Figure 10 C). This would indicate that more time spent in the arena does not equate to more Arc proteins expressed. These results give us confidence that the control groups used for each protocol are appropriate baselines for comparisons.

4.5 Additional Regional Analysis

This thesis is focused on neuronal activation in the CA1 region of the hippocampus because when cells in this layer are inactivated, object memory impairments are observed under the right conditions. In addition to this analysis, neuronal activation was also compared in the CA3 region of the hippocampus, dentate gyrus, and in the perirhinal cortex in the same sections that were used to analyze CA1. In these regions, two group comparisons were made: weak AH3 vs. weak Sample, and strong AH3 vs. strong Sample. Each regional analysis was conducted using an independent samples t-test totaling 6 analyses. No significant differences were seen between AH3 and Sample mice in either the weak or the strong memory protocol for CA3, DG, or PRh (Figure 11). Additionally, the data for each of the four regions for both the strong and weak memory protocols were combined and a 2x4 ANOVA was run as a more alternative analysis to compare each respective Sample session to its AH3 control session. This test revealed a significant interaction effect between brain region and memory condition ($F(9,114) = 4.61$, $P < 0.05$). Post-hoc comparisons using the Holm-Sidak test revealed that activation in the CA1 region of the hippocampus in Strong Sample mice was significantly greater than that of the CA1 region in Strong AH3 mice ($t(9) = 3.94$, $P <$

0.001), activation in the dentate gyrus was significantly greater for the Weak AH3 mice than the Weak Sample mice ($t(9) = 3.55$, $P < 0.01$), and activation in the dentate gyrus was significantly greater in the Strong AH3 mice than the Strong Sample mice ($t(9) = 3.28$, $P < 0.01$) (Figure 12). There was no significant main effect for brain region ($F(3,114) = 0.33$, $P > 0.05$) or for memory condition ($F(3,114) = 0.90$, $P > 0.05$).

PART V: GENERAL DISCUSSION

The present set of experiments was designed to elucidate the nature of hippocampal dependent object memory in the classic novel object recognition task in male C57BL/6J mice. The goal of these studies was to demonstrate hippocampal activity during two different variations of the NOR task using immunohistochemistry to stain for arc proteins which facilitate the encoding and consolidation of synaptic plasticity induced by learning and memory (Plath *et al*., 2006), allowing for the visualization of individual cells that were active during the experiment. The two variations of the task were used to test whether the formation of strong object memory or weak object memory, respectively, differentially recruits CA1 neurons. Analysis of brain sections extracted from these mice has shown that during the sample session of the strong memory protocol, a significantly larger ensemble of neurons was recruited in the CA1 region of the dorsal hippocampus when objects were present in the arena, as compared to when the mice were placed in the empty arena during arena habituation 3. This increase in active neurons during object exploration likely reflects the encoding of object memory representation within the hippocampus. This finding is in agreement with previous reports that when mice are allowed to explore each sample object for 30 seconds, the memory that is encoded for that object is dependent on an intact and fully functioning hippocampus (Cohen *et al.* 2013; de Lima et al. 2006; Hammond et al. 2004).

Furthermore, the analysis of brain sections extracted from mice in the weak memory sample session group has shown that the ensemble of active neurons within the CA1 layer of the dorsal hippocampus was not significantly different in magnitude from the ensemble that was active in the hippocampus of mice in the weak memory arena habituation 3 group during which mice were placed in an empty arena. These results suggest that although the sample group mice were placed in the arena with the sample objects and were allowed to freely explore them, the amount of exploration was not enough to encode a memory that was strong enough to be dependent on the hippocampus. These findings are in agreement with previous findings that have shown that when mice explored sample objects for a limited amount of time (in this case 10 seconds on each object), the recognition of those objects during the test session was not impaired by hippocampal lesion or inactivation (Winters *et al.*, 2008) but could be enhanced by SK channel blockade. It seems that when the object memory that is encoded during the sample session is a weak memory, it is supported by other structures within the medial temporal lobe, particularly the perirhinal cortex. These findings taken together indicate that somewhere between 10 seconds and 30 seconds of exploration of sample objects, the signal that represents object memory processing exceeds an unknown threshold that now includes processing done by the hippocampus.

Additionally, the data indicate that there was greater overall activation in the dentate gyrus in the AH3 as compared to the Sample mice in both the Weak Memory protocol and the Strong Memory protocol (Figure 12). This finding seems counterintuitive since it contradicts the results in CA1; however, this result is in concurrent with those published by Albasser *et al*. 2010, in which they reported that when

were exposed to novel objects in their paradigm, there was a significant increase in c-fos expression within the CA1 region that coincided with a significant decrease in expression in the dentate gyrus. This thesis emphasized the CA1 region of the hippocampus because that is the main output region of the hippocampus, and inactivating it produces clear impairment in object recognition memory. Further study is required to investigate this simultaneous decrease in dentate gyrus activation during novelty detection, but one possible explanation is that the detection of novelty activates a different circuit within the hippocampus then the classically studied tri-synaptic loop.

5.1 Lesion and Inactivation

It has been argued by some that the hippocampus is involved in object recognition memory, but that structures outside of the hippocampus such as the cortical areas in the medial temporal lobe are sufficient to support such memory in the absence of hippocampal function (Mumby *et al.*, 2007). Mumby et al. 2007 found that object recognition memory was impaired when NMDA lesions were induced after the encoding of the object memory, but not when the lesions occurred before the encoding stage of the memory. They concluded that if an object memory is encoded when the hippocampus is intact and functional, then retrieval of that memory depends on the hippocampus being intact and functional as well. Additionally, they concluded that if an object memory has been encoded in the absence of a functional hippocampus, then it can be retrieved without hippocampal function as well. This provides a possible explanation as to why previous findings using various techniques to lesion the hippocampus have found that the lesions do not impair object memory in the NOR task (Forwood *et al*. 2005; Kesner *et al.* 1993; Winters *et al.* 2004). In these cases, it appears as though in the absence of an intact

hippocampus, extrahippocampal structures, especially the perirhinal cortex, are capable of compensating for the fact that the hippocampus is not online to process object recognition memory.

It has been previously noted here that lesion studies are not always the most appropriate way to infer the function of a specific brain region, since this technique renders the region of interest destroyed, meaning it was never available to process incoming information it might normally have if the connections formed within the neurons remained intact. A technique that has proven to be quite a viable alternative is through temporary inactivation via bilateral infusion cannulae. This technique has provided support for the idea that the mouse hippocampus does play a critical role in both object memory consolidation and retrieval (Cohen *et al.* 2013). When infusions of muscimol to inactivate the CA1 region of the dorsal hippocampus were delivered before the sample session, object recognition was impaired during the test session 24 hours later. Similarly, infusions of muscimol before the test session also impaired object recognition memory. The current study provides further support for this notion using immunohistochemical techniques to show that a larger portion of the neurons within the CA1 layer of the dorsal hippocampus were active during the sample session as compared to control animals who did not get exposure to any objects.

While infusion techniques have proven to be an effective treatment to temporarily inactivate the hippocampus and have yielded promising results, these studies still draw a considerable amount of criticism. One of these criticisms regarding, in particular, infusions done in the perirhinal cortex is that the amount of drug that is infused into the region does not have a large enough area of effect to cause enough inactivation to

produce impairment, despite the fact that in the hippocampus, inactivating only about 1% of hippocampal volume is enough to produce severe object recognition impairments (Cohen *et al.,* 2013). The present study indicates that as a result of the high amount of exploration allowed in the strong memory protocol, the hippocampus is significantly more active than both AH3 groups and the weak sample group, which were not significantly different from each other respectively. The stain for the immediate early gene Arc in this case is justified by the fact that Arc knockout mice are unable to form long term memories (Tzingounis & Nicoll, 2006); disrupting Arc transcription in the CA1 layer of the dorsal hippocampus would likely cause impairments in object recognition memory due to an inability to consolidate long term memory. Future studies could continue to use inactivation techniques to further examine this question through the utilization of viral injection techniques such as optogenetics. This technique will allow us to selectively inactivate neurons in a specific region of interest literally like a light switch. Optogenetics utilizes a laser or LED light source to activate halorhodopsin, a light gated ion channel which can be expressed in neurons via injection of viral vectors carrying the halorhodopsin gene, to induce an inhibitory signal within all cells that are expressing this protein. In this way we can allow the mice to explore sample objects while inactivating cells only when the mice are actually exploring the sample objects. This technique could provide further support for our highly contested argument that object recognition is hippocampal dependent.

5.2 Contextual and Spatial Aspects of NOR

The whole premise of the current study and the related body of research hinges on the notion that the object recognition truly is non-spatial. Some argue that the objects in

the task are used as spatial cues indicating that when the novel object is present, the animal is processing the arena as a novel environment which would engage the hippocampus due to spatial and contextual processing. We can contend this point by looking at the distance traveled in Sample and AH3 sessions. If the Sample session mice perceived that the arena with the sample objects present was a novel environment, one would expect to see a significant increase in the movement throughout the arena, indicating their increased exploration of the new context. This, however, is not the case: mice in the AH3 sessions did not differ in distance traveled as compared to the Sample mice in both the Weak and Strong Memory protocols respectively.

Studies have tried to investigate this question and one in particular employed a Y-shaped arena for the NOR task, in which one object would be presented in each arm such that the object in the other arm would not be visible to the animal (Winters *et al.*, 2004). The authors argued that the utilization of this type of arena diminished the spatial and contextual components of the task. They found that rats with hippocampal lesions were not impaired in object recognition, while perirhinal lesioned rats had substantial impairments in the task. The authors concluded that the hippocampus is only involved in the NOR task in a context rich environment. However, one could easily argue that this unique kind of arena may have presented itself as a unique spatial environment. One major flaw of this study was that the experimenters did not control for the amount that the rats explored the sample objects. Another study attempted to get at this question from a different angle, running NOR with repeated Sample sessions, each in a novel context, 24 hours apart, in which the shape of the arena, texture of the floor, and the odor inside the arena were manipulated (Cohen *et al.*, 2013). These mice received intracranial infusions

of muscimol bilaterally in the CA1 layer of the dorsal hippocampus prior to the test session. The result was that control animals showed a preference for the novel object which was presented in a novel context, while the muscimol infusion group did not perform above chance. One would expect that if the mice perceived the test session objects as part of the context of the environment, the control mice should not show a preference for the novel object, since it only remembered the familiar object as part of the context of the sample arenas. This refutes the argument that the only reason hippocampal inactivations impair novel preference in the NOR task is due to the contextual component of the task.

Certain limitations remain in the methods used in the present study. Ultimately it can still be argued that this increase in hippocampal activation seen in the strong memory sample mice could be due to a perceived contextual novelty. The NOR task does not inherently offer a way to dissociate contextual information from object information due to the fact that no object can be observed in the absence of some form of context. Another limitation that comes with these methods is the fact that Arc positive counts made on sections taken from the brain in the region of the hippocampus represent general estimates of cells that were active, and do not indicate actual activation levels. In other words there is no technique that has been perfected yet that has the ability to quantify the actual number of active cells in a given region. It is also worth mentioning that the type of cell that is being labelled cannot be identified simply by visualization, thus, we cannot be sure if these cells being counted are pyramidal neurons, interneurons, or some other type of neuron that may or may not be processing object specific information. One way that we can investigate the identity of these cells is through a double-labelling study

staining for Arc and parvalbumin, a protein expressed by interneurons, or Arc and CAMKII, which is expressed in pyramidal neurons.

5.3 Object Recognition Memory in the Medial Temporal Lobe

The view that the rodent hippocampus does not process object memory, and is thus severely limited as far as its implications as an adequate model to emulate the human hippocampus is a common view held by many researchers and is one that is supported by a substantial amount of data (Albasser *et al.*, 2010; Winters *et al.*, 2008; Winters & Bussey, 2005). The bucket theory proposed by Cohen & Stackman (2014) is a qualitative or metaphorical model by which object memory may be processed in the medial temporal lobe. It posits that the perirhinal cortex and the hippocampus can be thought of as buckets and as the mouse is exploring an object, the perirhinal cortex begins to accumulate information about the object, pouring into the bucket. As exploration continues, the perirhinal bucket begins to fill with more information about the object until it reaches capacity. Once the perirhinal bucket has been filled, the information that was stored inside it spills over into the hippocampal bucket. At this point, with the object information being stored in the hippocampus, the object memory is no longer dependent on the perirhinal cortex, instead the memory is now hippocampal dependent (Cohen & Stackman, 2014). This model proposes a mechanism that concurs with the idea first suggested by Squire (2007) that the medial temporal lobe cortical structures are essential for weak memory processing, while the hippocampus is essential for processing strong memories.

5.4 Familiarity vs. Recollection

The role of the hippocampus in familiarity and recollection in object recognition memory has long been debated. The findings of Winters and Bussey (2005) showed that pre-sample, pre-test, and time-dependent post-sample lidocaine infusions into the perirhinal cortex of rats disrupts object recognition memory in the NOR task, providing evidence for the idea that the perirhinal cortex functioning supports familiarity memory (Winters & Bussey, 2005). Good *et al*. 2007 published findings that demonstrated that rats with hippocampal lesions were impaired in object recognition tasks only when the sample and the test session occurred in different contexts, suggesting that this form of object recognition can be classified as recollection because it required the rats to accurately recall the context in which the objects were first encountered. Among others who have studied this distinction is Sauvage *et al.* 2008 which reported findings that when the hippocampus is lesioned in rats, recollection memory is decreased while familiarity memory is increased, arguing that these processes are qualitatively different and that the hippocampus supports recollection and not familiarity. This finding in particular can be troublesome in that they are arguing that familiarity and recollection are qualitatively different processes that can be dissociated between the hippocampus and the perirhinal cortex, yet they report that familiarity memory increased as a result of their hippocampal lesions, which should not occur if these functions are truly dissociable.

Human studies have yielded slightly different results that suggests that the hippocampus can be involved in both familiarity and recollection. This line of research has led to an alternative conclusion about the familiarity-recollection distinction, that is the activity seen within the hippocampus and perirhinal cortex respectively is regulated

by memory strength instead of familiarity and recollection. Analysis of fMRI data and ROC curves of hit and false alarm rates on learning tasks has revealed a positive correlation between activity in the hippocampus and strength of the memory, as well as no indication that recollection memory is selectively supported by the hippocampus (Wais *et al.*, 2006; Shrager *et al.*, 2008). In the review of this literature, after looking at hundreds of studies done in humans, monkeys, and rodents, Squire *et al.* 2007 concluded that there was significant evidence for familiarity and recollection signals in both the hippocampus and in the perirhinal cortex, and that both the hippocampus and the perirhinal cortex are active during both kinds of recognition. The present study provides further support for this notion that the deciding factor in whether object recognition memory is dependent on the hippocampus or not is the strength of the memory that is encoded.

5.5 Conclusion

The present study lends support to the distinction of weak and strong memory and that the hippocampus supports strong memories which was originally proposed by Squire *et al.* 2007. In their review, they suggested that the differences seen in memory supported by the hippocampus and perirhinal cortex respectively, with the traditional view being that perirhinal cortex is responsibile for familiarity memory and the hippocampus is responsible for recollection memory, were actually better characterized by the strength of the memory in question. This idea suggested that medial temporal lobe structures neighboring the hippocampus were responsible for encoding weak memory as opposed to familiarity recognition. Furthermore, the present study provides evidence for a phenomenon that has been well characterized on the behavioral level, by showing

hippocampal activity on the molecular level. Increased hippocampal activation during the exploration of objects during the NOR task provides strong support for the case made by infusion and lesion data suggesting that the hippocampus is essential for object recognition memory.

The literature on novel object recognition in rodents is clearly divided into two camps; one that claims the memory for objects in rodents is processed exclusively by the perirhinal cortex, and the other that claims that an intact and properly functioning hippocampus is necessary for the recognition of a familiar object. Both arguments have reasonable merit to them and many explanations have been offered by both sides as to why the other's findings contradict their own. This study seeks to bridge the gap between the two opposing arguments by providing evidence that suggests that object memory processing is done by both the perirhinal cortex and the hippocampus in rodents, but that the dependence of the memory on each structure depends on the strength of the memory, or alternatively, the amount of information gathered about the object. By showing that strong object memories engage the hippocampus to a greater degree than weak object memories, we take one step closer to a unifying theory of object memory processing, while providing support for the rodent hippocampus as an appropriate model to study human learning and memory disorders.

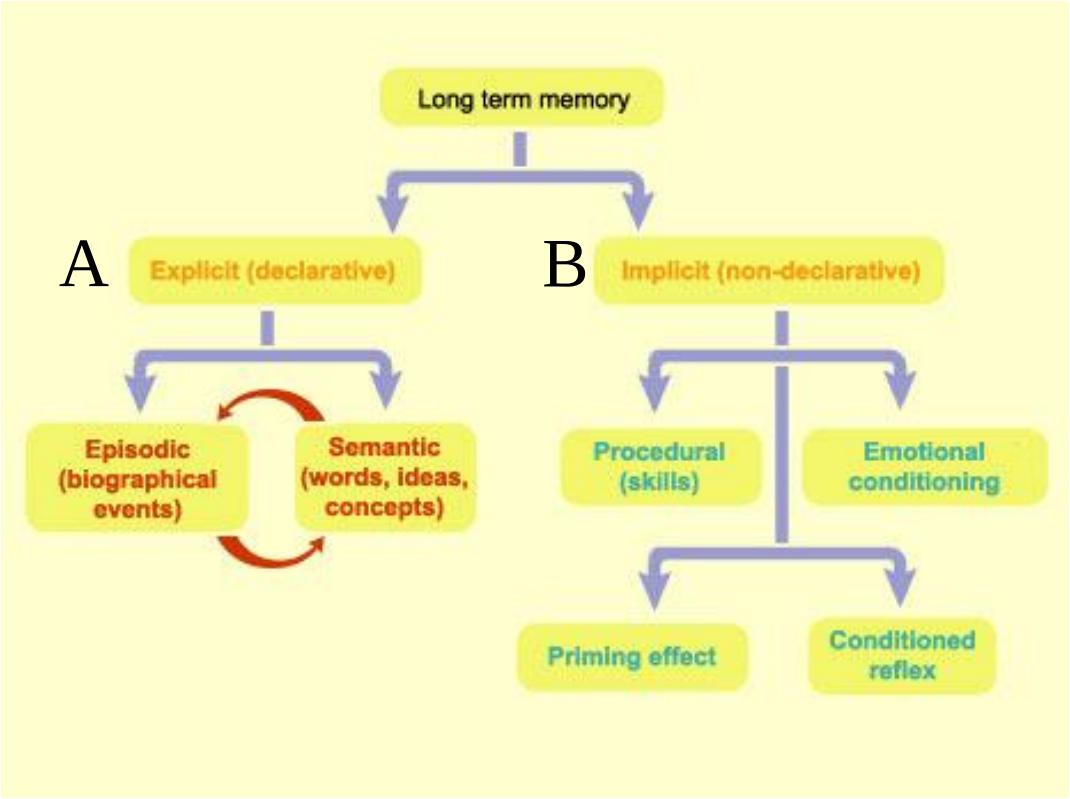


Figure 1 Long-term Memory schematic. **A.)** Declarative (explicit) memory (dependent on medial temporal lobe) organization. **B.)** Non-declarative (implicit) memory organization (dependent on structures outside of the medial temporal lobe.

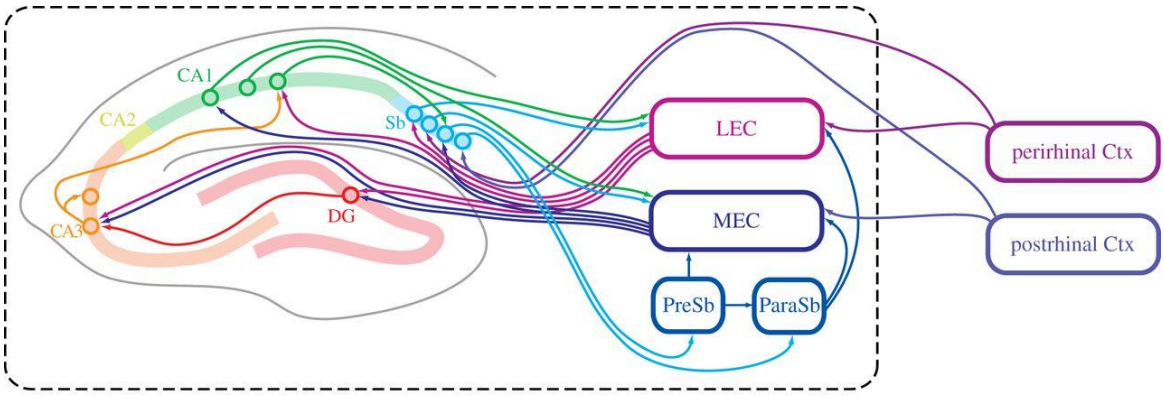


Figure 2. Diagram of hippocampal circuit and flow of information in the medial temporal lobe. (O'Keefe *et al.*, 2013)

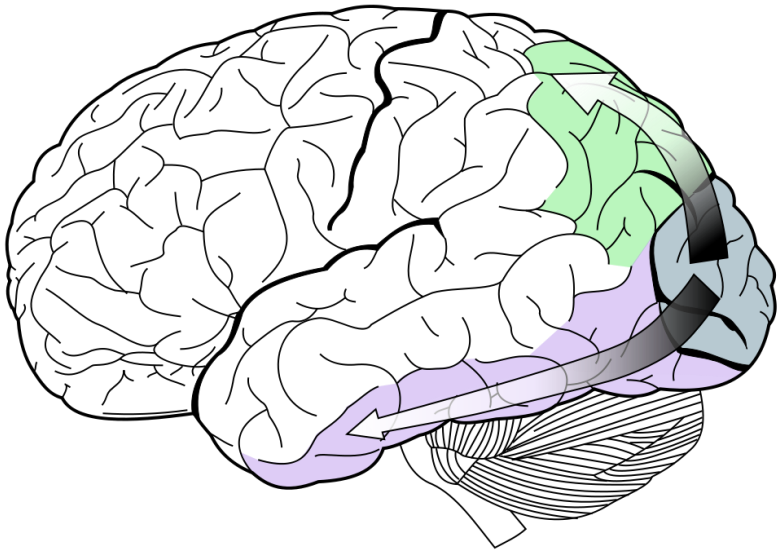


Figure 3. The dorsal and ventral stream compose the what and where pathways conveying sensory information that converges on the hippocampus.

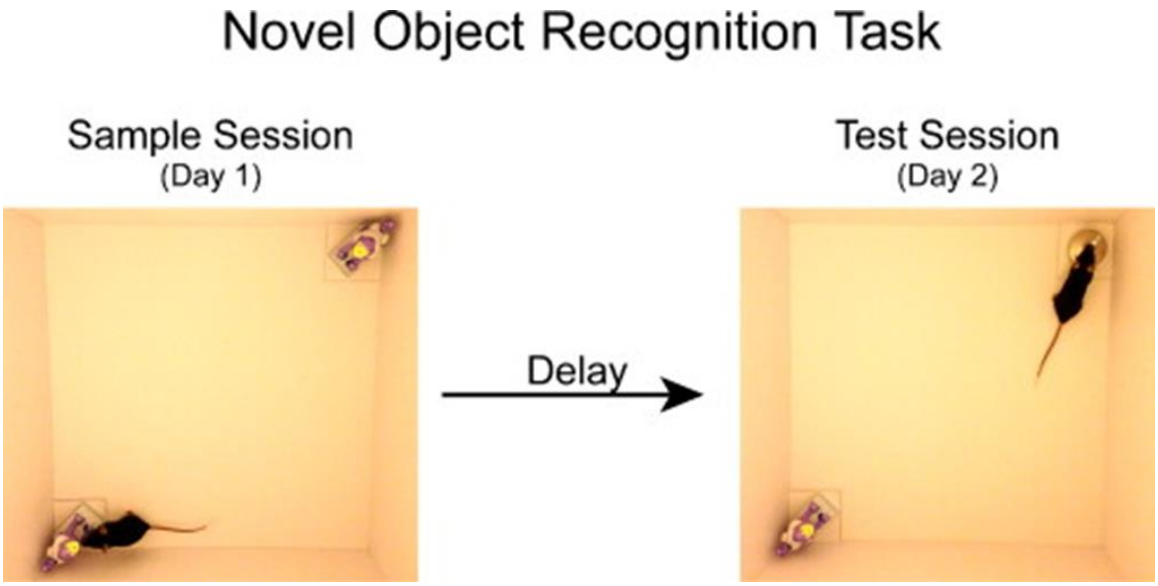


Figure 4. Classic Novel Object Recognition task for behavioral testing. During the sample sesioon, rodents explore two identical objects. After some delay the test session is given in which one familiar object from sample session and one novel object never seen before are explored. If memory from sample session is intact, the animal will spend more time exploring the novel object. (Adapted from Cohen *et al.* 2013).

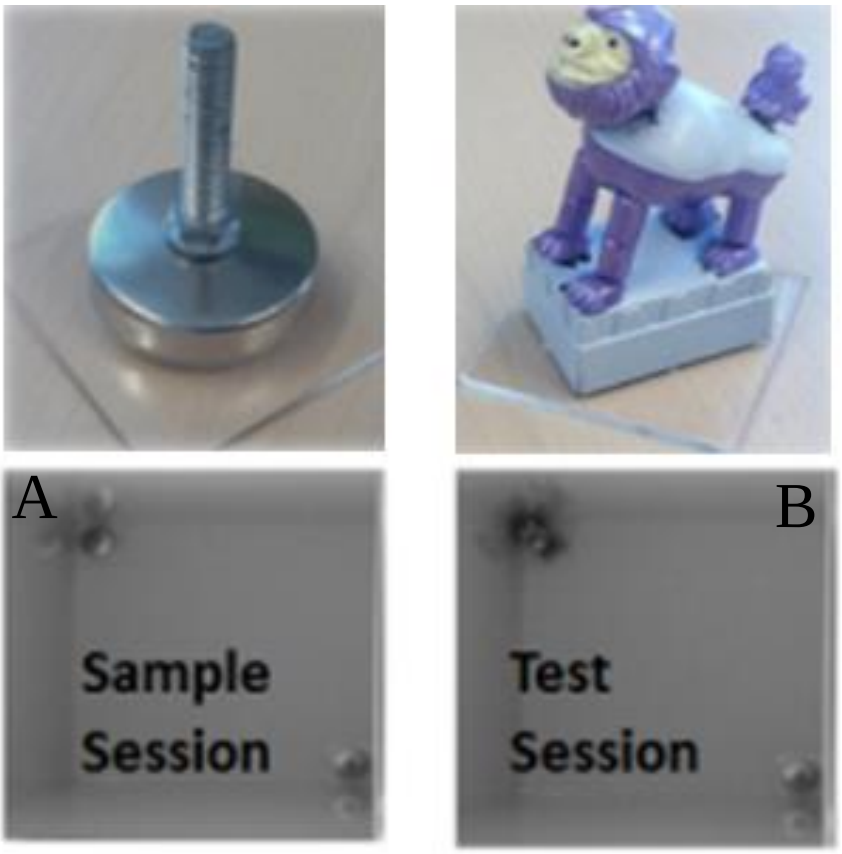


Figure 5. Images of 3D objects used for the sample session and test session; **A.)** refrigerator foot, and **B.)** plastic monkey

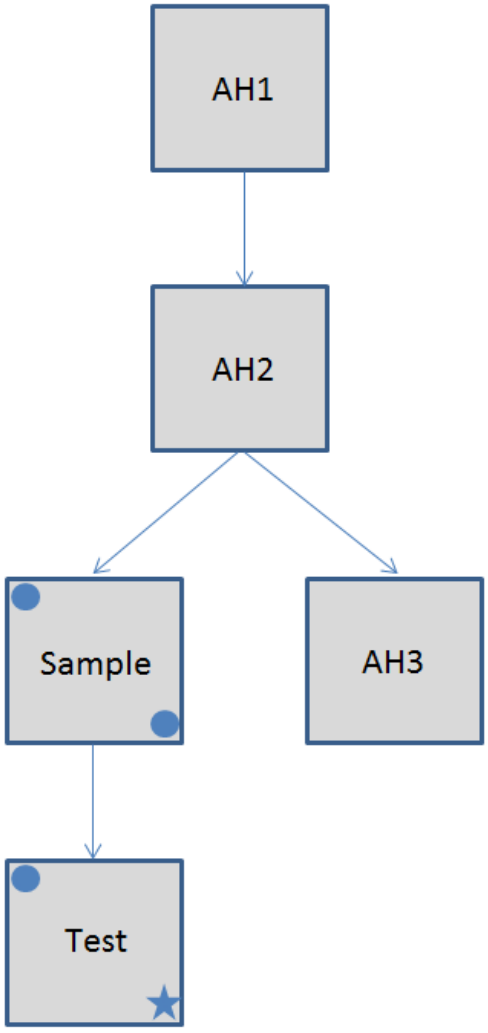


Figure 6. Novel Object Recognition task used for both the strong memory and weak memory protocols. Each arrow indicates a 24 hour delay period before the start of the next session of the task. Mice were divided into two groups following AH2, and euthanized following the sample session or AH3 respectively. A small cohort of mice who received sample session continued on to the test session to verify object memory was encoded.

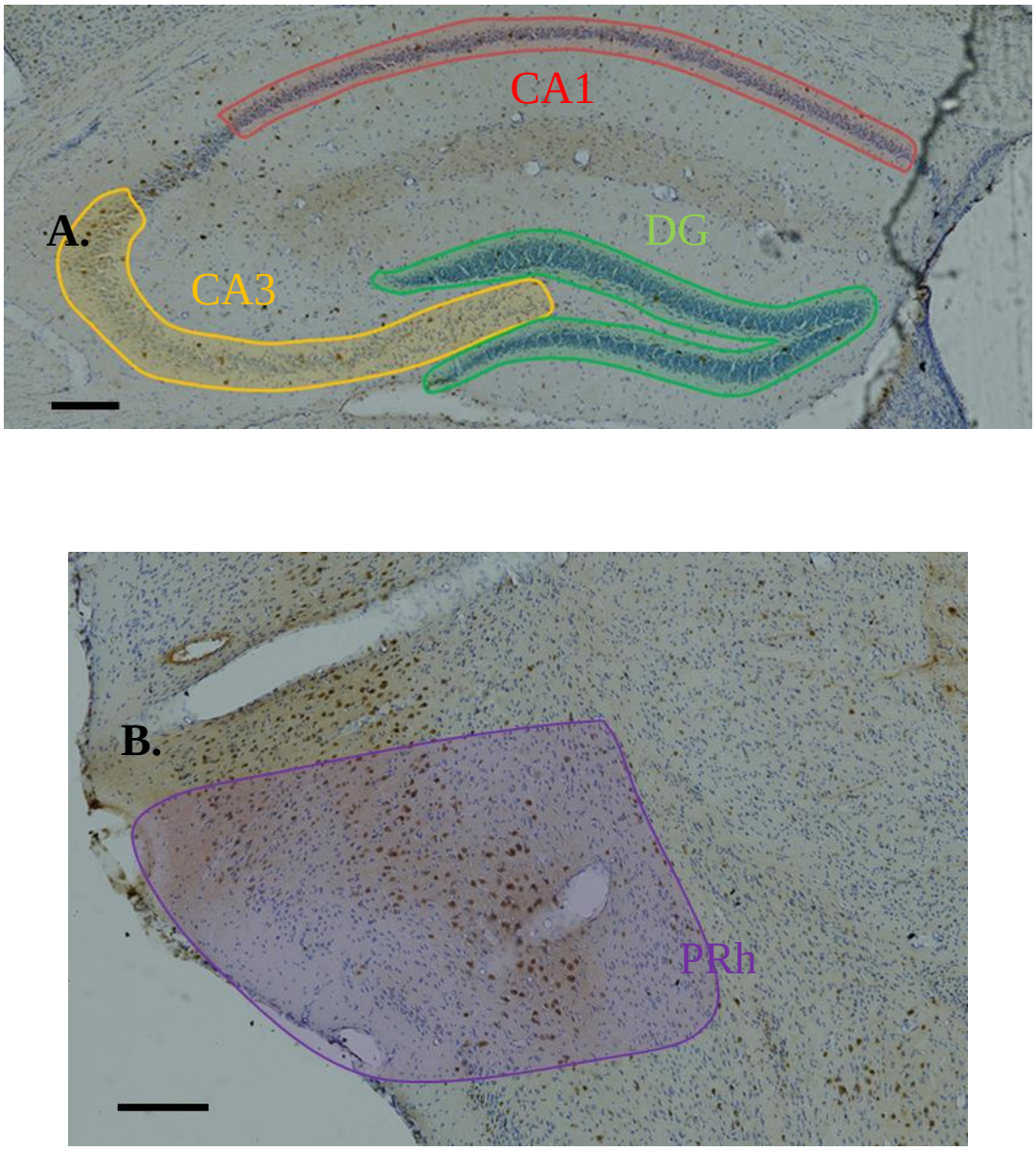


Figure 7. A.) Sample section of the hippocampus with subregions labelled and stained for Arc proteins. Neurons appear stained as blue while arc positive neurons appear as dark brown. **B.)** The perirhinal cortex on the same section is shown. Photomicrographs were taken at 40x magnification. Scale bar 100 µm.

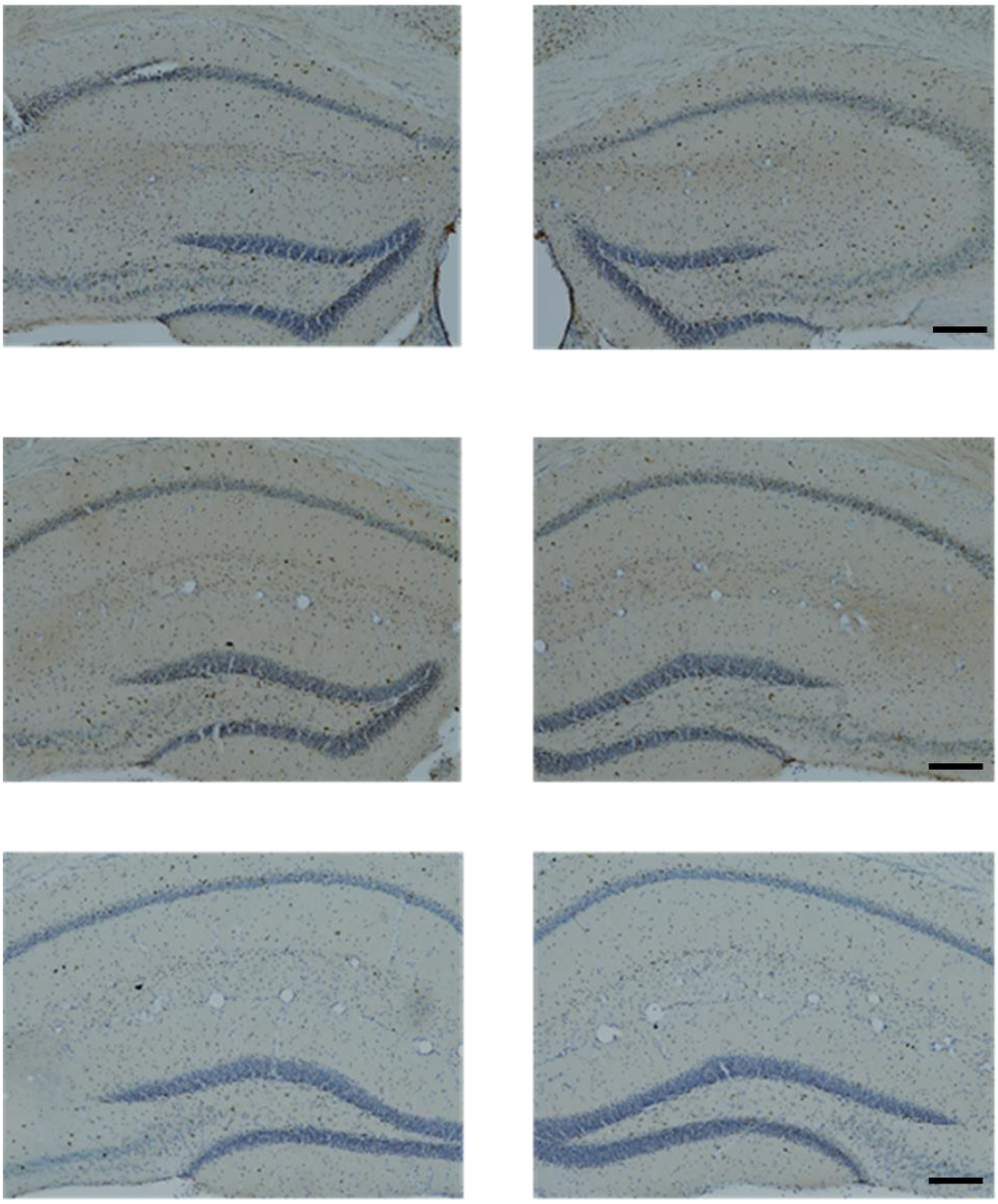


Figure 8. Hippocampal sections taken from the dorsal hippocampus of one representative mouse. Two bilateral sections from three different levels (dorsal, medial, ventral) of the dorsal hippocampus were matched and analyzed. Photomicrographs taken at 40x magnification. Scale bar 100 μm.

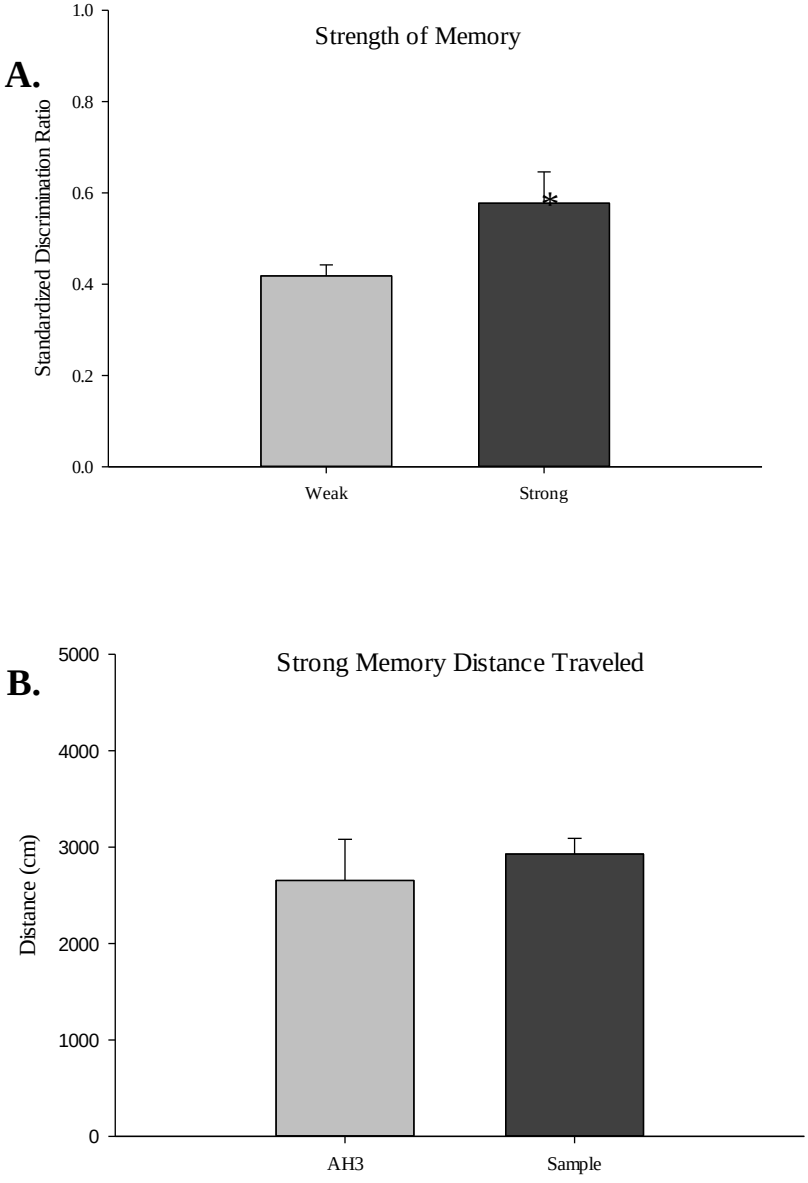
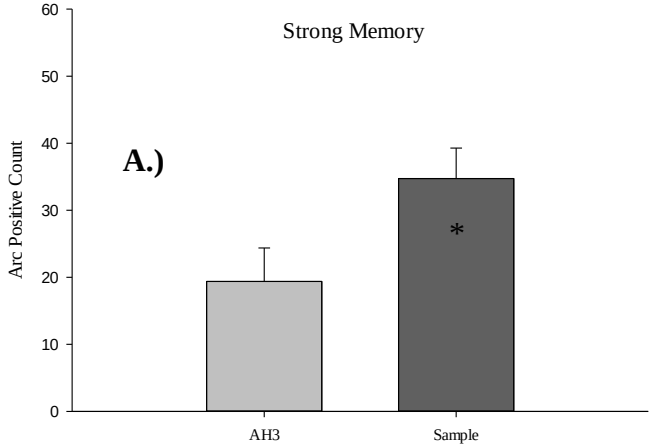
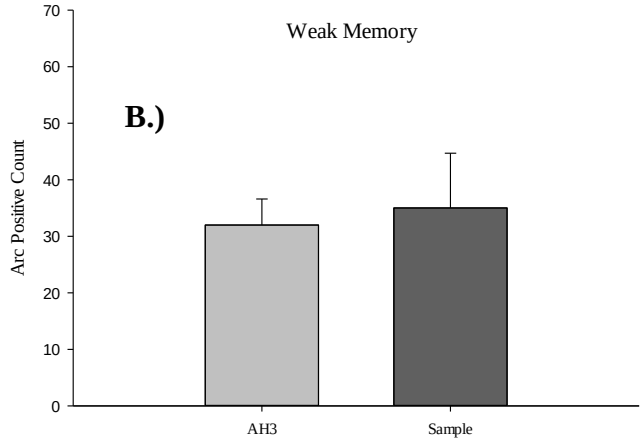


Figure 9. Behavioral data collected from test session mice show that **A.)** mice in the strong memory protocol encoded a stronger memory for the sample objects than the mice in the weak memory protocol as shown by a significantly greater preference for the novel object (*independent samples *t*-test, $P < 0.05$). **B.)** Distanced traveled in the strong memory protocol was not significantly different between the Sample and AH3 groups





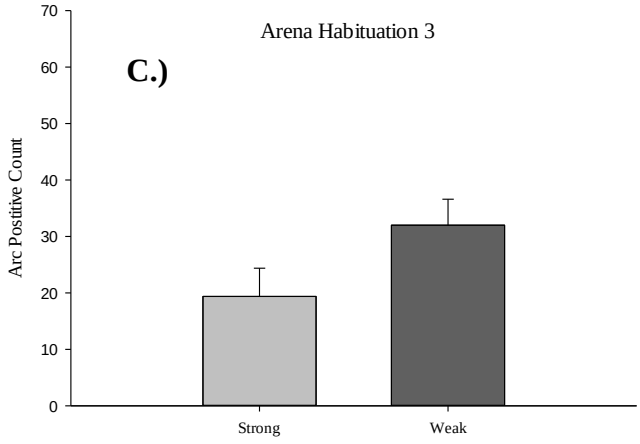


Figure 10. A.) comparison of mean arc positive counts in the strong memory protocol AH3 vs. Sample (*independent samples *t*-test, $P < 0.05$).

B.) comparison of mean arc positive counts in the weak memory protocol AH3 vs. Sample.

C.) comparison of mean arc positive counts of the AH3 session mice in the strong memory protocol vs. the weak memory protocol.

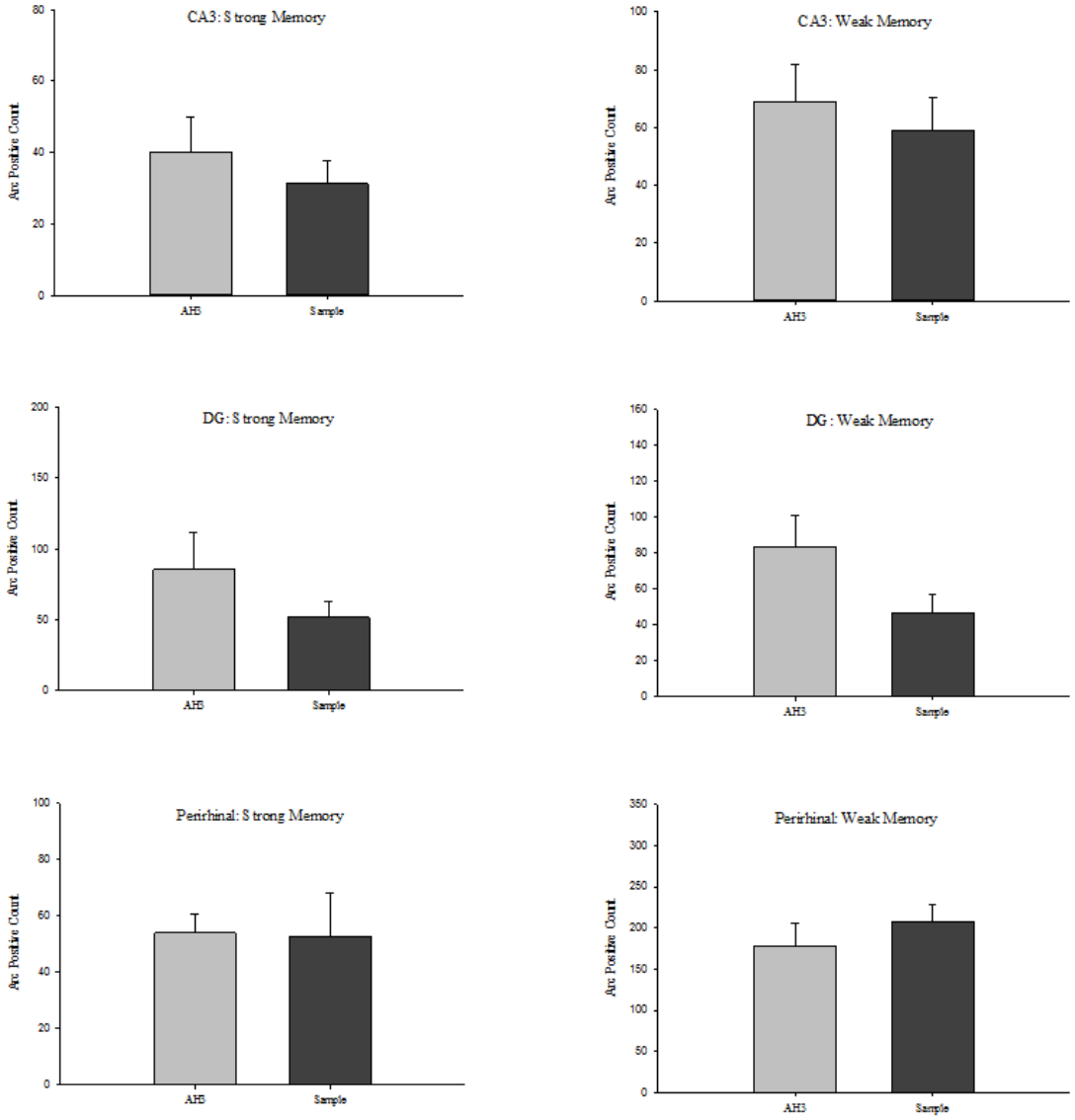


Figure 11. Analysis of Arc positive cell counts between Sample and AH3 mice in both the Weak and Strong Memory protocols in the CA3, dentate Gyrus (DG), and perirhinal cortex subregions did not yield any significant differences (independent samples t-test, all *P*'s > 0.05).

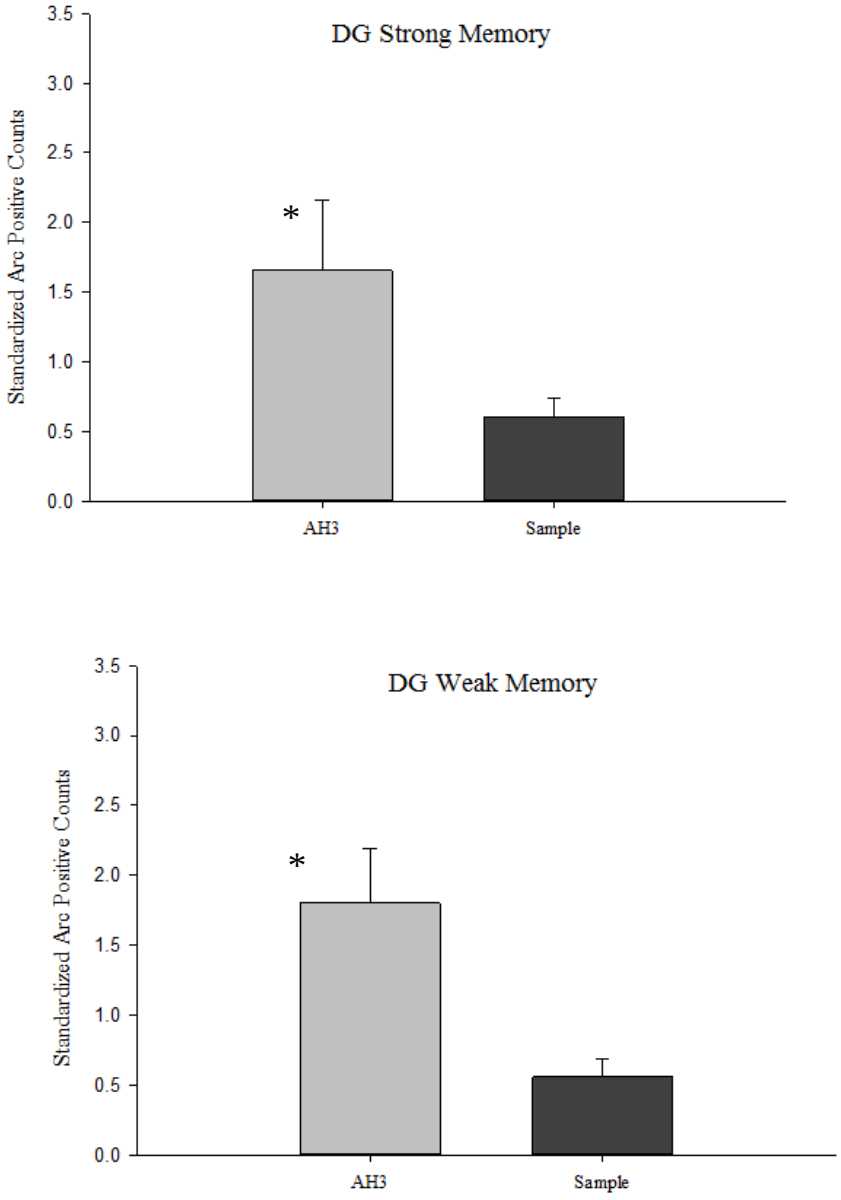


Figure 12. After standardizing counts to correct for normality, strong AH3 mice had significantly more activation in the DG than their Strong Sample controls. This difference was also observed in the Weak Memory AH3 mice when compared to the Weak Sample mice (*2 way ANOVA, $P < 0.05$).

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