

COVER SHEET

TITLE: Neuronal Ensemble Reactivation in the CA3 Hippocampal Region in Memory Form

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due to Pattern Completion

ABSTRACT

The hippocampus functions in many memory processes; not only is it essential to the storage of new memories, but the retrieval of associated memories as well. A postulate of memory recollection called pattern completion, thought to occur in the hippocampus, states that existing memories can be recalled using retrieval cues present at the time the memory was formed. The circuit level mechanisms responsible for episodic memory are considered a display of pattern completion within neuronal networks. This theory assumes memories are recalled when the cells in C3 activated by the original stimulus are reactivated with partial cues that stimulate a subset of the previously potentiated Activated Ensemble (AE) and in turn recall the original neuronal activity pattern. The major goal of this project is to determine if a partial cue that can evoke memory of the original event does lead to activation of the auto-associative neural network corresponding to the original event. We are interested in showing that the magnitude of the reactivation of the original neural network corresponds to the strength of memory for the original event.

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ABSTRACT

The hippocampus functions in many memory processes; not only is it essential to the storage of new memories, but the retrieval of associated memories as well (Giovanello, 2004). A postulate of memory recollection called pattern completion, thought to occur in the hippocampus, states that existing memories can be recalled using retrieval cues present at the time the memory was formed. The circuit level mechanisms responsible for episodic memory are considered a display of pattern completion within neuronal networks. This theory assumes memories are recalled when the cells in C3 activated by the original stimulus are reactivated with partial cues that stimulate a subset of the previously potentiated Activated Ensemble (AE) and in turn recall the original neuronal activity pattern. The major goal of this project is to determine if a partial cue that can evoke memory of the original event does lead to activation of the auto-associative neural network corresponding to the original event. We are interested in showing that the magnitude of the reactivation of the original neural network corresponds to the strength of memory for the original event.

Introduction

The hippocampus serves important roles in both learning and memory functions. One important function of the hippocampus is its ability to recall a full memory when only a portion of the original event is presented. The proposed mechanism for this process, termed pattern completion, is an important part of memory recall that has been described in detail by Rolls (2016; 2010). The neural connectivity within the hippocampus allows a group of neurons, an autoassociative network, to create a pattern representing a memory from which a cue can be used to recall the same neural network and retrieve the memory (Hunsaker, 2013). There is great interest in the mechanism of pattern completion and its contribution to memory functioning.

Pattern completion occurs due to the high degree of auto-associativity of the CA3 pyramidal cells (Rolls, 2010). The neurons active in CA3 during the original experience, called the active ensemble (AE), are reactivated during recall by means of long term potentiation (LTP)

in the CA3 synapses of the active ensemble during the learning event. It is thought that stimulating a fragment of the active ensemble of the original event, a partial cue, can activate enough of the active ensemble to recall the original event and this idea is supported by multiple experimental studies (Rolls, 2016). When the CA3 NMDA receptors were knocked out in mice performing a pattern completion test in a water maze, their ability to perform the task with partial cues was impaired. This test showed that NMDA receptor-dependent synaptic plasticity in the CA3 is crucial for pattern completion to occur in the hippocampus (Nakazawa et al, 2002). We want to determine whether neuronal activity in CA3 region displays pattern completion that is associated with the recall of an event when a rat is presented with partial cues from the original event in behavioral tests.

Recent studies show there is overlap between the cell ensembles in the CA3 activated during memory encoding and retrieval. Vazdarjanova and Guzowski (2004) placed rats in two slightly different environments 30-minutes apart and monitored the activation of ensemble neurons in the CA3 and CA1 using immediate early gene (IEG) brain-imaging. The overlap of the CA3 neurons activated in the two environments was much more significant than in the CA1, supporting the theory that the CA3 may play a major role in pattern completion in the hippocampus. Still, no research has demonstrated successful memory recollection in behavior tests with the existence of pattern completion during these events; the experiments we have been conducting are aimed at this.

Our experiment aims to understand the role of pattern completion in memory by examining whether the activated ensemble from a learning event is reactivated in the CA3 region during recall of the event from degenerated input. We directly examined the ability of a rat presented with a portion of the original event to recall the full original event, and if

corresponding pattern completion is observed in the CA3 region. If this is shown, it would support the theoretical postulate of the cellular mechanisms responsible for pattern completion, and would be a huge advance in the understanding of the mechanism of memory recollection. If it does indeed occur, the issue becomes what level of partial cues are necessary for pattern completion to occur. This is dependent upon the connections in the CA3, and these connections are determined by strength of LTP between synapses in the hippocampus and its intrinsic connectivity. In a recent publication that used a realistic model, presentation of one-third of the original input could provoke reliable, robust pattern completion (Guzman, 2016). Through behavioral analysis, and ultimately gene analysis, we hope to determine what percent of the original input must be given by the partial cue so that the original memory is recalled.

Experimental Approach to the problem

A rat's hippocampus serves as the main cognitive system for the formation and mapping of spatial memory; in fact, multiple studies determined that object-place-context recognition can only occur within the hippocampus (Mumby, D.G. as cited in Albani 2014). Memory tests such as Morris water maze or contextual fear conditioning produce robust results, but they do not assess the natural mechanisms of pattern completion because the animal is influenced by the inputs of the Amygdala (Lee and Kesner, 2004; D' Hooze, 2001). Our experiments focused on creating a natural memory test without the use of fear or other strong emotions so solely hippocampal neurons will be activated and studied.

We tested pattern completion in two trial object- location memory tests (OLM), a stress-free experiment testing the role of only the hippocampus without input from any other areas to avoid influencing or disrupting pattern completion. According to Albani (2014), rodents have a natural desire to explore new objects; we took advantage of this preference in our memory tests.

Our experiment analyzed rats' interest in familiar and novel object locations to test the capacity of their object- place-context recognition using graded cues. With the presence of sufficient partial cues, pattern completion would be triggered and the rat would recognize that one object was located in a novel position in the recall test. The basis for the memory test is that the rat would be more interested in the novel object than the familiar object if sufficient memory was recalled.

Pattern completion implies the reactivation of neurons active at the time of learning; consequently, measuring the overlap of activated neurons in learning and memory recollection serves as a measure of pattern completion. Following the behavior portion of the experiment, cellular activities in CA3 hippocampal regions were measured using immediate early gene (IEG) activations as markers. Pattern overlaps were measured as the strength of partial cues varied, and the significance of the pattern overlaps was tested by correlating them with the memory seen in the OLM experiments. The basis for these experiments comes from similar experiments done by Vazdarjanova and McNaughton using the same IEGs, Arc and Homer 1a (H1a). Using fluorescence *in situ* hybridization, the experiment demonstrated that Arc and H1a expression is strongly induced in the same neurons of rat hippocampus after exploration of a novel environment. The results supported the view that Arc and H1a are activated during novel experiences and function in long term potentiation; thus, their activation serves as a marker for neuronal activation during memory encoding and recollection (Vazdarjanova et al, 2002). The actual level of neuron activity that corresponds to Arc or H1a induction has not been quantified, but because they are Calcium activated genes it is assumed to be substantial (Vazdarjanova et al. 2006).

Not only can a greater understanding of memory processes enhance our ability to learn and improve our ability to recall what we have learned, but it can also be crucial in the understanding of many diseases that impair memory such as Alzheimer's. Those with Alzheimer's have difficulty recalling memory without strong inputs, which may be a result of failures in pattern completion. Evidence from mouse models of Alzheimer's suggests that poor memory retrieval, not storage, is the cause of memory loss. Any further understanding of the mechanisms of pattern completion could create new treatment options for this disease (Roy et al, 2016). The results of this experiment could establish the behavioral conditions in which pattern completion plays a role in memory while simultaneously measuring cellular pattern completion.

Methods

Subjects and study area

This experiment used Sprague Dawley and Long Evans rats ranging from 3-6 months old and weighing an average of 300 grams. The rats were kept in cages, two per cage, in a room with a 12-hour light/dark cycle and were removed from the room during the dark period to increase their activity. At all times during the experiment and between trials the rats had unrestricted access to food and water.

The experiments took place in a plastic arena (23.9×24.1 inches) lined with sawdust and covered on all sides so that the rat could not see out of the arena. Two identical objects,

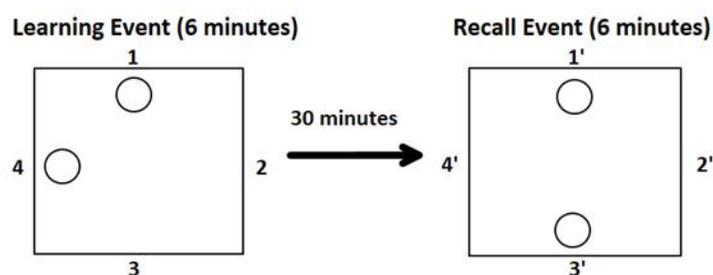


Figure 1: This figure represents the behavior experimental setup. Circles denote objects and numbers 1-4 denote different cues. *These cues may or may not be present in the recall event, depending on the experiment.

varying between experiments, were taped down so that the rats could not move them (as shown

in *Figure 1*). Color of objects and cues was insignificant as these rats did not have particularly color-sensitive vision. The cues used consisted of black designs on white paper, one on each side of the arena. The rats were habituated as seen in Vazdarjanova (2004) to allow them to adjust to human interaction and to the experimental arena.

Test details

The rat was brought into the room in one covered cage and put into a different cage. The rat was given 30 minutes in the cage, uncovered, to adjust to the light. The objects were placed adjacent to or across from each other and all cues placed onto the sides of cardboard (*Figure 1*). The arena and objects were cleaned with ethanol to remove odors the rat may have detected from previous experiments. The rat was taken close to the experimental arena to adjust to light for two minutes. It was removed from its cage and put in the arena for six minutes for the learning test, during which time the experimenter left to avoid interfering with the rat. After six minutes, the rat was removed from the container and placed back into its cage for another 30 minutes before the recall test. The arena and objects were cleaned with ethanol (Vazdarjanova, 2004), sawdust replaced, one object moved, and a varying number of cues removed. The same steps were repeated in the changed experimental arena on the same rat for the recall test.

Each experiment entailed the displacement of one object (the novel object) in the recall test and the removal of a specific number of cues. The control experiment was the removal of no cues, and other experiments consisted of the removal of one, two, three, or all four cues. Video recordings of each trial were viewed in order to measure the amount of time spent smelling, rubbing against, and examining each object (climbing on the object was ignored) in both the learning and recall test. An analysis of this data served as a measure of memory. A memory ratio (MR) was calculated and defined as $MR = (y-x)/(y+x)$, where y was the time spent at the novel-

location object during recall and x was the time spent at the original object during recall. The bias corrected memory ratio, which accounted for object bias in the learning experiment, was calculated and defined as $MR' = MR/r$ where r was the ratio of time spent at the novel and original object during learning. For the memory ratio, a value of zero showed no preference, positive MR showed a preference for the novel object, and negative MR showed a preference for the original object. For example, if the rat spent twice as long exploring the novel position, $MR = 0.33$, showing robust memory. If all time was spent with novel object, $MR = 1$. Any trial for which the time at either object during the learning test was 25% greater than or less than the mean was excluded due to object bias.

An analysis of the memory ratios reflected the memory of object placement. An ANOVA test was run on data to test their significance. Due to the varying nature of the behavior experiments by rat, we created a linear regression model that accounted for all the discrepancies between experiments using SAS software in order to more accurately test the significance of our data. A Tukey-Kramer test was then run on the data in order to determine the significance of difference between each experiment.

IEG Protocol

The rat was sacrificed five minutes after the end of the recall phase of the experiment. Once the rat was sacrificed, the hippocampus was removed from the brain and frozen, then later cut into 16micron slices using a cryostat machine. The Activated cell Ensembles (AE) during the learning and recall phases were located using cellular compartment analysis of temporal activity in situ hybridization (catFISH) with RNA probes for the immediate early genes (IEG) ARC and HOMER1a. These genes are activated during intensive cell activity, as occurs during behavioral events (Kubik et al, 2007). These methods consisted of first creating a riboprobe with either

Digoxygenin for ARC or Fluorescein for HOMER1a using a standard MAXIscrip procedure (Ambian, 2008). After probes were created they were hybridized to their respective RNA strands on the hippocampal slice, following the description by Guzowski (2001). Once hybridization was complete, the tissue was washed and reacted with antibodies to both Digoxygenin and Fluorescein that were conjugated to Horseradish Peroxidase (HRP). After washing the tissue, it was reacted with both a Cyanine-3-TSA substrate kit and with a Fluorescein-TSA substrate kit. These have distinguishable fluorescence spectra so can be individually identified by fluorescence microscopy. Each cell the nuclei was labeled with YOYO-1 to see a clear representation of the nucleus.

Future experimental procedures

Using a confocal microscope, the presence of mRNA from each IEG will be analyzed in the nucleus and in the cytoplasm of cells of the hippocampal CA3 region. This analysis method will allow for the visualization of which neurons were activated during both the learning and the recall events within the same neurons. The mRNA for ARC appears within 2 minutes of the event and remains in the nucleus for only 5-10 minutes (Guzowski, 2001). If ARC is visualized in the nucleus of a cell, this will signify that that cell was active during the recall phase. HOMER mRNA requires 45 minutes to be synthesized and remains in the nucleus for 15-30 minutes after activation (Guzowski, 2001). Thus, if HOMER1a is visualized in the nucleus of a cell, that cell was active during the learning phase of the experiment. Cells active during learning and recall phase will exhibit both ARC and HOMER1a mRNA in the nucleus. The extent to which nuclear ARC and HOMER1a overlap will be a measure of the extent to which the recall ensemble reproduces the learning ensemble.

Results

The data from the experiments conducted showed that there was a correlation between

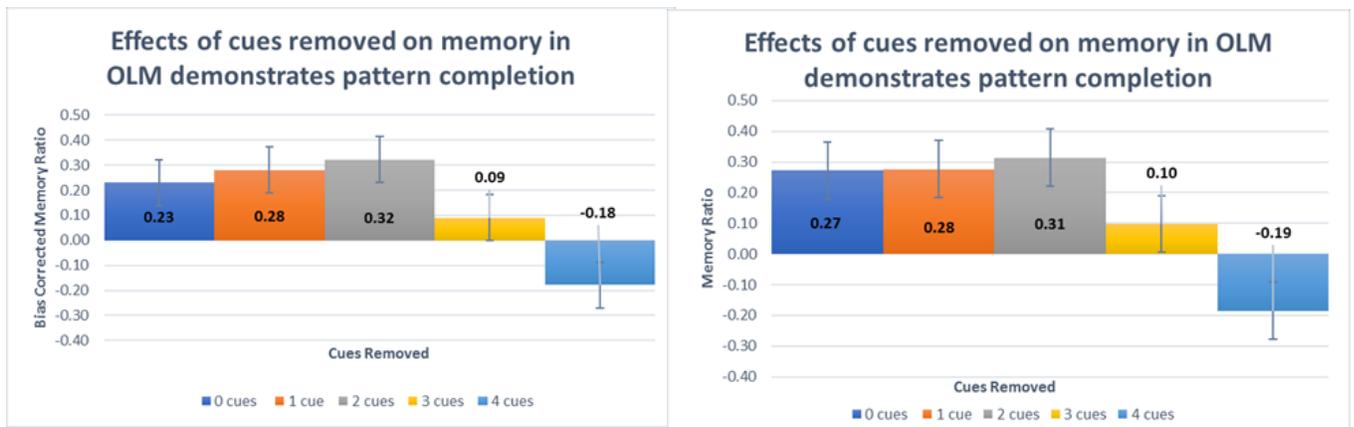


Figure 2: These graph displays the average Memory Ratio and average bias corrected memory ratio for each OLM experiment with graded cue removal. These data show positive memory ratios, thus memory, for the removal of 0, 1, and 2 cues. The removal of 3 cues show a significant decrease in the memory ratios and memory, while 4 cues removed showed negative memory ratios suggesting no memory recollection. Bias correction resulted in slight changes to the memory ratio and more accurate results.

the strength of the cues present and memory ratio. Figure 2 shows that the removal of zero, one, or two cues provided sufficient cue strength for memory recollection, evident from the positive memory ratios. However, the removal of three cues significantly decreased the rat's memory ratio; this decrease in memory ratio represents the rat's lack of memory and impaired pattern completion. The similar average memory ratios seen in experiments with both one and two cues removed indicate that the difference between the strength of three or two cues does not substantially affect the rat's ability to perform pattern completion. As shown in figure 2, experiments with four cues removed were the only tests in which there was a slight preference for the familiar object. These data suggest that the strength of cues provided had to be greater than one partial cue in order for pattern completion to occur.

An ANOVA test on the Bias Corrected Exploration Ratio yielded a P-value of 9.37E-07, indicating our data was very significant. Using our SAS model, we were able to determine that all of our data for zero, one, two and four cues was significant with P-values less than 0.05, but data for three cues removed was not significant (p value= 0.479). This suggested that we needed a larger sample size for future experiments and to collect more data. Using a Tukey-Kramer test, we were able to establish that there was a significant difference in memory for zero and four cues removed (P-value= 0.0063), one and four cues removed (P-value=0.0217), and two and four cues removed (P-Value=0.0163). Moreover, memory for zero, one, and two cues removed was significantly different than four cues removed, while three cues removed was not significantly different from either (table 1). These statistical analyses of our data suggest that zero, one, and two cues removed allows for sufficient pattern completion while removing all cues inhibits memory recollection.

Expected Future Results

To date, we have prepared the DIG labeled probe for ARC and are now carrying out the ISH studies. The next step will be to correlate the memory in the behavior tests with the neuronal reactivation overlap in the CA3 hippocampal region. Cells active during learning and recall phase will exhibit both ARC and HOMER1a mRNA in the nucleus. Figure 3 shows hippocampal slices before undergoing florescence *in situ* hybridization, as well as hippocampal cells after a catFISH experiment from another study. The extent to which nuclear ARC and HOMER1a overlap is a measure of the extent to which the recall ensemble reproduces the learning ensemble. Increasing overlap is expected to be associated

Tukey-Kramer Grouping for cues Least Squares Means (Alpha=0.05)		
LS-means with the same letter are not significantly different.		
cues	Estimate	
0	0.1611	A
		A
2	0.1361	A
		A
1	0.1236	A
		A
3	0.04200	B A
		B
4	-0.1457	B

Table 1: Cues refers to cues removed. Different letters signify statistical differences in the data. 0, 1, and 2 cues removed are statistically different from 4 cues removed.

with a higher memory ratio and therefore the degree of IEG overlap will follow the same pattern. We expect to see strong overlap when zero, one, or two cues are removed during recall and much less overlap when recall is with three or four cues removed. We are currently unable to predict the level of overlap that will signify good or bad memory, but it will likely be based on an analysis of the correlation with the memory tests. If a correlation is found between the degree of overlap and the memory ratio, this study will prove very important in experimentally establishing the concept of pattern completion in memory.

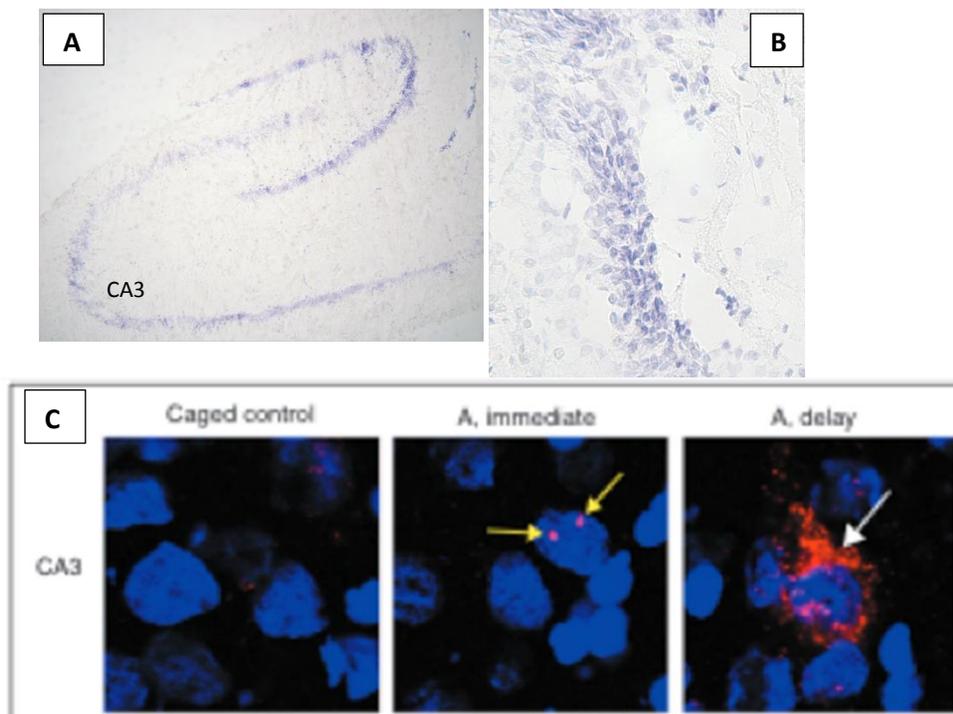


Figure 3: **A-** Photo of a hippocampal section stained with cresyl violet, viewed from a microscope at 4X. **B-** Photo of CA3 neurons in the hippocampus, stained with cresyl violet, viewed from a microscope at 40X. The nucleus is stained darker than the cytoplasm in the cells. **C-** Fluorescence micrograph of CA3 cells in rat hippocampus that have been in situ hybridized with antisense mRNA for ARC. Nuclei are stained blue. There is very little ARC in caged controls. Immediately after 5 minute cage exploration, ARC is in the nucleus. 30 minutes after cage exploration (delay) all the ARC is out of the nucleus, in the cytoplasm. From Guzowski (2001).

Discussion

The statistical analyses show that our memory ratio was a valid measure of the rat's ability to use partial cues to recognize when an object was moved to a novel location; thus, the memory ratio was indeed an appropriate measure of pattern completion for a behavioral study. The positive memory ratios for the presence of four, three, and two cues showed that a rat can adequately perform object-location memory tests with the presence of at least 50% of cues from the original event. No statistically significant differences were found between the removal of zero, one or two cues, hence similar levels of pattern completion were exhibited in these experiments. However, these experiments were significantly different from those with the removal of all four cues, for which there was a negative memory ratio close to 0 (Figure 2 and 3). Consequently, we were able to prove experimentally that the removal of all four cues inhibited pattern completion.

Gilbert (2009) highlights the idea that autoassociative networks in the hippocampus, especially the CA3 region, are responsible for paired association learning of an object and its location. When neuronal ensembles in the CA3 are activated they allow the rat to recall the memory of the original event if the strength of the cues is sufficient for the rat to associate the object and its original location. Experimental data from our study supports these theories behind pattern completion. Rats were able to pattern complete successfully with zero, one, and two cues removed. One possible explanation for the similar memory controlled exploration ratio with zero, one and two cues removed is that the hippocampus tends to de-emphasize any changes to the norm encountered in the novel situation during pattern completion (Vazdarjanova, 2004). Consequently, the difference between the presence of four, three, and two cues present was not significant enough to change the hippocampus's ability to pattern complete. Removing four cues

from the experimental arena impaired ability to pattern complete, which supports the idea that the strength of the input directly relates to the ease with which the hippocampus is able to retrieve the full activated ensemble formed in the learning event. It is evident from these findings that not just any partial cue may be used to retrieve the memory; cue strength must be robust to fully stimulate the activated ensemble of the original event.

The removal of three cues did lower the memory ratio substantially, but the statistical insignificance of the data suggests that more experiments must be conducted to support this. Moreover, because the experiments removing three cues were not significantly different from the other levels of cue removal, we are unable to accurately state the effect of 75% cue removal on pattern completion. This is a critical point experimentally because as shown in a recent study, one third of cues present may be a threshold at which the hippocampus's ability to pattern complete changes (Guzman et al, 2016). These findings are supported by the findings of Vazdarjanova (2004), whose experiments demonstrated that if the partial environmental cues were not strong enough, the "threshold for remapping" was not met and thus pattern completion did not occur. It will be very important in future experiments to have statistically significant data for the removal of three cues so that we can successfully quantify the effect of graded cues on pattern completion.

From these findings, many important aspects of memory and pattern completion become evident. In learning processes, it is necessary to have a substantial strength of partial cues in order to fully retrieve an existing memory. This can be applied to all aspects of learning; for instance, to remember material learned in a class there must be a significant amount of the initial material learned present during testing. Significant partial cues were necessary for object-placement recognition; thus, partial cues must be present in order for our brains to recognize

familiar locations, things, or even people. This may be significant in treating Schizophrenia or other mental illnesses that can interfere with memory retrieval. Therapy that utilizes partial cues in order to recall memories may increase the activity and overall functioning of the hippocampus, which long-term may improve neurological functioning.

We are currently continuing with the IEG catFISH studies. Without any results at present, we are still unable to establish the experimental conditions in which pattern completion is seen on a cellular level in the CA3 region of the hippocampus. Given our data from behavior

experiments, we can predict that we will see significant overlap of IEGs in experiments in which 50% of partial cues are present during recall if the proposed neural mechanisms are correct.

Figure 4 represents the expected relationship between memory ratio and cellular overlap: an

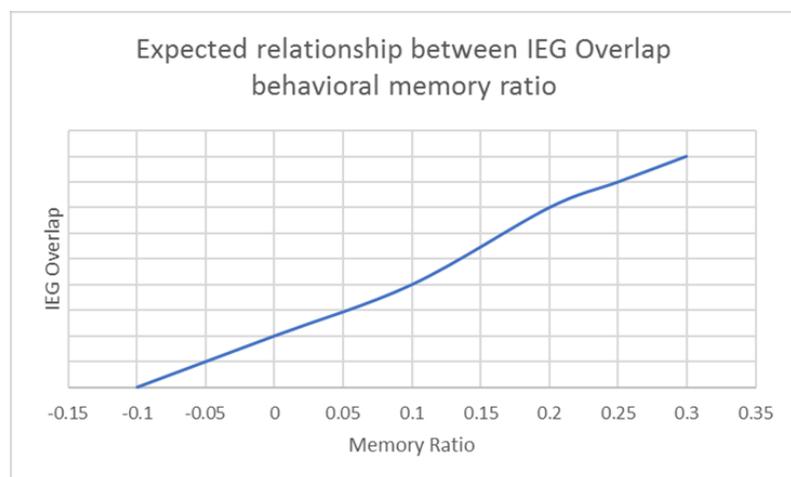


Figure 4: This figure indicates our expectations for future studies. We predict that as the memory ratio increases, indicating full pattern completion, IEG overlap will increase accordingly.

increase in IEG overlap with increasing MR. If pattern completion occurs by some other neural mechanism, we may not see a correlation between memory in behavior experiments and IEG overlap in the CA3. In this case, future studies of pattern completion would need to focus on developing a more accurate model for memory recall.

After the results of this experiment are complete, we will conduct variations of the experiment to gain a fuller understanding of both pattern completion and its effect on the overlap

of activated ensembles. We will continue to study this topic by introducing an intervening experiment between the learning and recall event to discover if recall can persist even with a disrupting event. From this, we hope to understand how much deterioration can there be in reactivation before the memory is corrupted. Preliminary data show that an intervening stimulus yields significantly different results from OLM test without an intervening stimulus. With the results from this study and future studies, we will establish conditions in which pattern completion is involved in memory recall. Pattern completion is the basis of memory recollection in the hippocampus, it is how we remember. A better understanding of the neural mechanisms of pattern completion is absolutely critical in improving the understanding of memory and could lead to the treatment of memory disorders.

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