DISTINCT CONTRIBUTIONS OF THE NUCLEUS REUNIENS,
PREFRONTAL CORTEX, AND HIPPOCAMPUS TO SPATIAL WORKING MEMORY

by

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The memory of my grandmother, Marilyn (Malka) Niedober. This survivor of the Holocaust, in the Warsaw Ghetto, lived to be 98 years old with 6 grandchildren and 3 great-grandchildren. She was the strongest, bravest, most remarkable person I have ever known, who shaped within me all of the traits I hold most dear.
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ABSTRACT

Memory is an abstract concept referring to stored representations of previous experience that may prove relevant to future behavior. Working memory refers to the process by which those representations are put to use in a given context, with a necessary temporal component. Spatial working memory (SWM), in particular, refers to those working memory activities occurring within the context of spatial navigation behaviors. The process requires representations of learned experiences, current context, comparisons between the two, and rule-based decision making. Each of these has been previously shown to depend on some combination of three critical structures; the prefrontal cortex (PFC), the hippocampus (HC), and the midline thalamic nucleus reuniens (Re). However, it had not yet been demonstrated when during the SWM timeline the information flow across specific pathways linking the structures was necessary to support accurate goal-directed navigation. The current experiments use optogenetics to target pathway-specific activity during isolated portions of the SWM timeline. The results, suggesting high-degrees of selectivity to contributions necessary to SWM, are discussed within the context of how the brain supports the construction and application of critical neuronal representations for: learned experiences, current context, comparisons between the two, and rule-based decision making.
Chapter 1

CIRCUIT DYNAMICS SUPPORTING SPATIAL WORKING MEMORY

1.1 Spatial Working Memory

There is an interesting thought experiment in which one attempts to describe a thing they have never experienced; an objective we quickly realize is impossible. Ultimately, our behavior is equally limited by representations of our experiences. Despite our ability to be imaginative and creative, our thoughts and actions are still restricted by representations of a world we have already experienced. The process by which animals create these representations of past experiences is called learning.

Simplistically, new bits of information enter into perception, by way of sensation\(^1\). That information is compared against encoded representations of other previous experiences, and the novelty is evaluated\(^2\). Perhaps the objects in a scene are new, but the scene is familiar. Perhaps the emotional context of this repeat experience have been altered by circumstance. The representations for previously experienced objects, places, contexts, episodes and more are called memory. Like astronomy, memory is a phenomenon that has been studied, in some fashion, for at least 2,000 years\(^3\), and likely contemplated for far longer. Systematic investigations have led to the grouping of behavioral assays of memory into a wide range memory types; episodic\(^4\), associative\(^5\), declarative\(^6\), etc.

Arguably, the utility of memory is in guiding behavior. Episodic memory, the memory for events, such as burning one’s finger on a fire or for being warned by their parents not to touch, is particularly useful for informing our decision of whether or not
to reach out and grab a burning log. We can reason that there are numerous overlapping cognitive constructs involved in this seemingly simple experience. The senses are activated by touching the log and that information must be transformed into a common communicable signal\(^7\), such as a neuronal spike train and an inflammation response. The various components of that signal have to be processed, and its characteristics perceived and encoded. Memory for previous experiences, direct or indirect, with similar characteristics are retrieved and evaluated\(^8\). A plan of action, or inhibition, must be formulated and communicated; such as to let go of the log and not pick it up again. The plan must be executed. If there is a mandatory delay period between any of these phases, the information must be maintained until it can be used\(^9\). The period during which all of these processes take place is generally referred to as working memory.

Working memory is a measurable behavioral construct in which an animal is actively engaging neural representations of a past experience to help evaluate the current context and guide behavioral responses\(^10\). Naturally, due to sharing a common set of languages, humans can relatively easily study this phenomenon in other humans. However, certain neuronal activity patterns, signatures of the neural representations of past experiences for instance, can currently only be collected using non-human animals; creatures we cannot be certain understand our languages. Thus, to determine how neurons are constructing, storing, and utilizing information to guide behavior, we have to explore working memory paradigms that non-human animals can perform.

We can imagine measuring working memory without exchanging information verbally. A taxi driver, for example, likely holds a mental map of the city in which they work. They pick up a fare and are given a destination. They might then encode
contextual information, such as their current location and the traffic at that time of
day. That information is compared against representations of past experience, like
their mental city map and expectations of traffic flow. A contextually-dependent plan
for navigating to the destination is formulated and initiated, with the likelihood for en
route updates; at all phases, a spatial map must be maintained in working memory.
This is a prime example of spatial working memory (SWM). As these tasks do not
require the use of human language, they are ideally suited for animal species for which
navigation is a fundamental behavior. Thus, SWM tasks can be used to collect and
analyze complex neuronal data from freely-behaving non-humans animals, allowing
researchers to ask and answer compelling questions about common, memory-related,
neural processes.

Before attempting to associate specific neuronal processes with various
components of SWM, we can define the components. We can reason that one possible
way to segment SWM is to consider space, working, and memory as distinct
components. Space can refer to demands on aspects of spatial cognition. This can
include representations of the view-from-above (allocentric) Cartesian coordinates
being occupied by the animal on an imaginary X-Y plane, as well as navigation along
various vectors within that space. Thus, in considering neuronal processes relevant to
SWM, we can investigate activity in structures associated with representing space. The
working component to SWM can refer to representations of time. SWM paradigms
necessarily incorporate a delay, requiring the linking of streams of contextual
information across a finite period of time. Therefore, we are implicating structures
that demonstrate activity associated with the representation of time. Finally, the
memory component can reasonably refer to complex cognitive abilities associated with
the sequencing of events; encoding previous experiences, learning their contextual importance and associated implications, and devising schema for how to interact with similar features. We can then focus our attention on local neuronal dynamics and circuit interactions associated with any, or all, of these components; namely, the prefrontal cortex (PFC), the hippocampus (HC), and the midline thalamic nucleus reuniens (Re).

1.2 A Tri-regional Circuit

The central nervous system (CNS) can be organized on numerous different anatomical scales. Most grossly, we can identify the spinal cord and the brain. The brain, in turn can be divided into the hindbrain, midbrain, and forebrain. We will focus on three critical forebrain structures, one of which resides in the thalamus and the others which reside in the cerebrum; the Re, PFC and HC.

The two cerebral structures of interest to the current studies are located within the temporal and frontal lobes; the HC\textsuperscript{13} and PFC\textsuperscript{14}, respectively. The most direct route for bi-directional communication between the PFC and HC seems to be through a small and highly selective midline thalamic nucleus, the Nucleus Reuniens (Re)\textsuperscript{15–17}. Thus, while it has been understood for some time that not every thalamic nucleus simply acts as a relay\textsuperscript{18–20}, only recently has there been increasing attention given to the role of certain thalamic nuclei in supporting complex cognitive processes.

In rats, dense direct projections from the ventral HC terminate directly within the mPFC\textsuperscript{21,22}. Though, recently, a sparse direct projection from the mPFC to the HC has been identified in mice\textsuperscript{23}, no such projection have yet been identified in any other mammal. There must be a way for the mPFC and the HC to dynamically and efficiently communicate. Continually accumulating evidence is suggesting that their
communication may depend heavily on the Re\textsuperscript{16,17}. The Re sends projections to both the mPFC and HC\textsuperscript{15,16}. Axons from both the mPFC and the HC enter into the Re\textsuperscript{24}. A subpopulation of the Re neurons that receive projections from the mPFC also project to neurons in the HC stratum lacunsum moleculare (SLM)\textsuperscript{17}. Most interestingly, a very small subset of Re neurons (~4-8\%) send bifurcating collaterals to both the mPFC and the HC simultaneously\textsuperscript{25}. Thus, we implicate a tri-regional, mPFC-Re-HC SWM circuit.

1.3 Neural Substrates of the Spatial Component

Focusing first on the space component of SWM, we can consider how the structures in this tri-regional circuit support spatial processing. In 1971, a groundbreaking study identified tuning curves within the firing patterns of a subpopulation of HC pyramidal neurons\textsuperscript{26}. In any given recording environment, approximately 30\% of HC pyramidal neurons will fire when an organism is at a specific location in an environment. These cells are referred to as place cells and the location specific firing is a place field. If we imagine an overlay of Cartesian coordinates onto a given environment, select neurons will fire maximally at a particular set of coordinates. Furthermore, these neurons remain relatively silent until the animal is near those coordinates. They will increase and decrease their firing rates as the animal gets closer to and further from the center of the field. Subsequent research has demonstrated that these firing patterns are not influenced by the direction from which the animal approaches the field, suggesting the role of a population of these place cells in constructing an allocentric cognitive map of the whole environment\textsuperscript{27}. Moreover, the size and shape of place fields along the dorso-ventral (top-bottom) and medio-lateral
(middle-side) axes are different\textsuperscript{28}, indicating that HC spatial representation is systemic and diverse.

Decades of research have since converged on the notion that HC spatial representation is critical to navigation and other cognitively demanding behaviors\textsuperscript{11,29}. Furthermore, the evidence tends to be consistent across species. To select one compelling report, there was an innovative analysis attempted to show that HC units, given a certain environment, were not simply tuned to place\textsuperscript{30}. Rats were trained to alternate on a T-shaped maze, requiring that they maintain a representation of their current and previous location; or, at the very least, a recognition for having recently been to a given side of the maze. Firing rates on the common portion of the maze distinguish between when the up-coming trajectory was to the left, while others preferred a right-bound trajectory.

In more recent years, attention has been given to cross-species investigations of HC neuronal dynamics in representing space. One particularly interesting species, for example, is bats. One study took advantage of the behavior bats, navigating in 3D space\textsuperscript{31}. They reported that, while flying, bat HC neurons were overwhelmingly involved in representing place fields that had a spheroidal structure with an epicenter located at coordinates along 3 spatial axes. In a subsequent report, the same group recorded from the HC of two distant species of bats while they crawled along 2 dimensional surfaces. They showed evidence of neurons with place fields, head direction tuning, and a conjunctive representation of the two features\textsuperscript{32}. Some place cells would continue to fire outside of the well-defined field, giving these spikes the appearance of noise. However, closer investigation revealed that, often times, these neurons were only spiking outside of the field when the head was oriented in
particular direction, suggesting a conjunctive representation of space-by-direction. The representation was directed at a target goal location in space\textsuperscript{33}. 

Simultaneously recording activity from large populations of single HC units and local field potentials (LFP) from the HC has also bolstered the evidence that HC spatial tuning is likely functionally relevant. Oscillations have been shown to group together spatial experiences\textsuperscript{34} and sequence place fields, both pro- and retrospectively\textsuperscript{35}. Traversal across sequential place fields is represented by sequential firing of place cells, locked to the periodicity of background 7-12 Hz oscillations\textsuperscript{36}. Sequential place fields overlap, such that several place cells may be active to varying degrees depending on the location of the animal relative to the respective epicenters of the overlapping fields\textsuperscript{34}. The authors reported that place field sequences can be compressed into single theta cycles. These so-called theta sequences differentially extend ahead of and behind the animal depending on its acceleration. These findings suggest that place field sequences are not simply representative of current space, but are actually synchronized into chunks that may be behaviorally relevant. 

In addition to the theta oscillation, there is another HC LFP trace heavily implicated in functionally organizing HC unit activity and sequencing place cells. The event is called a sharp-wave ripple (SWR) complex because it is a conjunction of a large negative deflection and several cycles of notably fast (150-250 Hz) ripples. SWRs have been shown to organize a sudden burst of spiking in a population of neurons, called population burst events (PBEs)\textsuperscript{37}. Careful analysis of which neurons spike, and in what order, has been shown to represent a replay of recently traversed place fields in a tightly compressed and ordered sequence\textsuperscript{38–42}. Researchers continually implicate this HC LFP signature in navigation and consolidation of recently traversed
space. If place fields represent not just space, but an event that occurred within that space, then perhaps some SWR-associated PBEs represent remote environments not currently occupied by the animal. Locking an analysis of firing rate to the already-known position of an animal can be restrictive in that it makes an assumption about the spatial coding of the observed spiking in representing the current environment. One recent study reported an alternative method, in which the transitions between a sequence of states was analyzed prior to considering the spatial location of the animal, and only then used to decode position. Additionally, they were able to identify compressed sequences of states, rather than place per se, during SWR-associated PBEs, offering an entirely new approach to considering how the spatial feature may only be an anchor for a more abstract representation of what occurs in that space.

Another structure continually demonstrated to support spatial processing is the PFC. In one study, researchers recorded mPFC activity, from both the prelimbic and infralimbic (p/il) subregions of the mPFC while rats navigated to a reward in a given environment. They reported that mPFC neurons also demonstrated spatial tuning reminiscent of place cells. Interestingly, more mPFC place cells had fields around the reward than anywhere else in the environment, suggesting that the spatial tuning represented salient target destinations. In another study, mPFC neurons were recorded while an animal investigated an object in an open field. One of several findings suggested that when object and place were associatively paired, mPFC neurons displayed spatially sensitive firing patterns. In a report with similar behavioral implications, rats were trained to navigate the various arms of a maze, based on associations between sampled objects and the location of those objects. They found that when the mPFC was suppressed, accurate navigation performance was impaired.
These findings suggest that there is a clear spatial component to how the rat mPFC represents goal-directed information.

Similar evidence of spatial tuning has been reliably reported in both non-human \(^{46-48}\) and human primates \(^{49,50}\). In one study, monkeys were trained to target spatial locations with goal-directed reaching in a visuomotor task across several trials \(^ {47} \). Prefrontal neurons appeared to have preferential firing rates for given spatial locations on given trials with only rare overlaps between trials. These findings suggest that mPFC neurons are goal sensitive, coding particular spatial information. In a study of the human PFC, participants interacted with a virtual spatial environment while sitting in an fMRI scanner. Changes from baseline in oxygenated blood content in the PFC represented an orderly and topographically organized map of the virtual environment \(^ {50} \). Among these structures was the lPFC, the functional and anatomical analog for the rat mPFC.

Spatial tuning has also been reported in the activity of Re neurons. There are neurons with tuning curves selective to the radial orientation to which the head of the animal is directed; “head direction cells” \(^ {51} \). Investigators recorded Re neurons while a rat was navigating an open field. They found that a subpopulation of neurons selectively increased their firing rates as the head was angled toward a particular direction, regardless of the current position of the animal in space. In another experiment, rats were trained to alternate on a T-shaped maze. Similar to HC “splitter cells” \(^ {50} \) that show distinct firing rates for left and right traversals, a subpopulation of Re neurons preferentially fired during a given left- or right-bound trajectory. Thus, a clear degree of spatial tuning was demonstrated in Re firing patterns. When Re is perturbed by [], the stability of recorded HC place fields depends on an intact Re \(^ {52} \).
These findings indicate that the output of Re activity influences the spatial tuning of downstream structures.

### 1.4 Neural Substrates of the Temporal Component

Another critical component of SWM is a sensitivity to time. The working component to SWM indicates that information representations must persist across a delay period that separates the sampled information from the target behavior across time. To support SWM, local and/or interactive activity within and between the HC, mPFC, and Re must demonstrate temporal coding. Two possible processes are by representing the passage of time or by rhythmically timing related coding processes with LFP oscillations.

One particularly prominent oscillation within the HC is the theta band. HC theta has been heavily implicated in both navigation and memory. In one study, theta was shown to time the alternation between environmental maps represented by place cell activity, given that place cells can map different locations in different environments. Neurons recorded from the lateral septum, a structure that is directly downstream of the HC, showed no direct spatial selectivity, but the timing in which the neurons spiked relative to HC theta phase coded for space. In an earlier study, the phenomenon of theta phase precession was shown to apply both to sequential spatial and non-spatial representations. Pyramidal neurons within the HC spike at particular moments during a given theta cycle, referred to as theta locking. However, theta phase precession occurs when firing sequences are condensed within a single theta cycle. The condensed sequence starts locked to the later phases of the theta cycle. As the animal continues through the task, spikes shift when during a theta cycle they prefer to spike to increasingly early phases. Additionally, other HC LFP
oscillations, like the gamma band (30-100 Hz), have also been associated with orchestrating functional interactions between structures\textsuperscript{59–61}. Collectively, these studies of oscillatory population dynamics in LFP traces suggest that the timing of information flow is critical to the function of the HC.

Time cells are neurons that demonstrate a rate code (i.e., information is provided by the timing of each spike) tuned to elapsed time. In one study\textsuperscript{62}, rats were trained to run a T-shaped maze. However, this maze had a treadmill in one portion of the common stem. As the rats ran through the task, they would arrive at the treadmill, and be confined for a controlled and varied delay period. The rats would continue to run, but space was dissociated from the running behavior as they were held constant by the treadmill. The authors reported that a subpopulation of HC pyramidal neurons would increase and decrease their firing rates as the animals traversed through time, but not space. The length of the delay period could be decoded based on the sequencing of time cells. Thus, there are HC neurons that directly represent particular moments in the passage of time.

Time is represented in multiple ways in PFC activity. Neuronal activity evolves over the time course of a task in reliable patterns. In one study of the primate PFC, macaques were trained to associate two stimuli across time\textsuperscript{63}. A visual stimulus was linked with a behavioral response across a delay period. The authors reported that PFC neurons responded distinctly to the onset of a stimulus as compared to the latest portion of a delay period, just prior to the expectation of a decision, suggesting a sensitivity to the delay period duration and a representation of time. Another display of temporal processing by PFC neurons can be found in the representation of ordered stimuli, as sequences are naturally a function of time. Investigators reported that when
the order of the stimulus presentation is relevant to guiding goal directed behavior, delay period firing rates could be used to decode the order\textsuperscript{64}.

Prefrontal neurons in primates are also tuned to time\textsuperscript{65}. A subpopulation of PFC neurons reached a maximum firing rate at precise times during the delay period of a visuomotor task, suggesting that the sequenced activity of these neuronal populations may countdown to the end of the delay period and the start of a behavior. Furthermore, a wealth of evidence suggests that neurons in the PFC entrain to LFP signatures with the HC to organize their timing during various tasks\textsuperscript{66–69}. Collectively, the evidence is overwhelmingly suggestive of the criticality for carefully calibrated timing mechanisms within the local activity of our tri-regional circuit, as well as in organizing the dynamics of their interactions.

1.5 Neural Substrates of the Memory Components

Having demonstrated that our tri-regional circuit supports both temporal and spatial coding, we can describe their contributions to memory-dependent tasks; the third component of SWM. One study of the mouse mPFC\textsuperscript{70} combined optogenetic manipulations with electrophysiological recordings to show that the activity of the mPFC during the delay period of a working memory task was critical to learning the rules of the task. The activity of particular proteins within the PFC, implicating not only physiological but molecular mechanisms, in a mouse model of Parkinson’s Disease was associated with deficits to working memory and deficient synaptic plasticity when compared to wild type mice\textsuperscript{71}.

Sustained activity during the delay period of a working memory task has been primarily reported in primates, during which the duration of the delay period typically only lasted for a few milliseconds. When investigators started to use rodents to study
SWM, they found that involvement of different structures, and the involvement of their interactions, varied dramatically with differences in the length of the delay period. At very short time delays, for instance, animals with either a lesioned PFC or a HC were still able to perform a given SWM task\textsuperscript{72,73}. When delay periods were relatively long and the sampled context could not be immediately used to guide behavior, lesions to the PFC impaired choice accuracy\textsuperscript{73,74}. Therefore, if information maintenance across the delay is a function of sustained neuronal activity in the PFC, a lesioned PFC should have been sufficient to disrupt performance. Additionally, the delay length should not be a contributing factor. There has to be an alternative set of mechanisms, with alternative timing, by which these structures were supporting SWM.

One striking example of HC activity supporting working memory was an extension of the “splitter cell” findings\textsuperscript{75}. Rats were trained to navigate a T-shaped maze on what is called the delayed non-match to position (DNMP) task. During the task, there is a context sampling period that is linked to a choice period across a delay. To capitalize on the tendency of rats to alternate during navigation\textsuperscript{76}, they were trained to link a the trajectory during a sampling traversal with a freely-chosen opposite trajectory to receive the reward. Recording HC pyramidal neurons, the authors reported that neurons did not simply demonstrate preferential firing rates when either a left or right trajectory was pending. Instead, firing rates distinguished between the first and second of the paired traversals. This suggested that there must be a mechanism for keeping track of the timing of the behavioral contexts during a SWM task; that HC neurons are distinguishing between the period of sampling contextual information and that of applying it to inform behavior.
The contributions of the HC and PFC each to various facets of learning and memory have been heavily investigated. Yet, the most compelling evidence for how they support the active use of learned representations to inform behavior comes from probing the mechanisms underlying their interaction. In one such study, researchers recorded from both the PFC and HC of rodents during sleep periods that followed task training. They extended earlier demonstration that slow-wave sleep (SWS) was critical for consolidating earlier experiences by hosting neuronal replays of those experienced event sequences. In fact, their data indicated that the reactivation of neural patterns suggestive of event replay in the PFC occurred during synchronized bursts that largely coincided with SWR events in the HC. In another report of PFC-HC synchrony, the input of the ventral HC (vHC) to the mPFC were suppressed during various portions of a SWM task; disruptions to this input during context sampling were associated with both disruptions to synchrony and to choice accuracy.

The critical role of mPFC-HC interactions during SWM, and their anatomical connectivity discussed earlier, is what has directed attention to the Re during SWM. To select one example, during a SWM task, several measures of mPFC-HC synchrony appear to be heightened while the animal navigates through the task. This study showed that, during the delay period preceding correct alternations, mPFC neurons were reliably entrained to the ascending phase of HC theta cycles. On correct alternation trials, HC and mPFC theta cycles, mPFC theta and gamma cycles, and mPFC gamma and HC theta cycles were all reliably phase locked as the rat approached and occupied the decision point (the T-junction). Most critically, infusing muscimol into the Re (thereby suppressing activity by activating local GABA<sub>a</sub> receptors) appeared to disrupt synchrony and decrease choice accuracy. The collective
demonstration is that each structure in the tri-regional circuit, as well as their interactions, supports all three necessary components of SWM.

1.6 Organizing the Spatial Working Memory Components into a Timeline

Based on the utility of memory in guiding behavior, and our definition of SWM components, we can imagine a logical information processing pipeline; a SWM timeline followed in each trial. First, there must be the construction of a representation for the task. The animal must be sufficiently exposed to the rules of the task to have built a usable and reliable approach for solving the task. This is the process by which a general background memory is formed; they have learned how to behave to find a reward.

Next, on any given trial, there is a phase during which the current context is being sampled, so that it might inform subsequent behavior. This context-sampling phase first requires that an active representation of the features of the environment be constructed. This must be accomplished through the acquisition and processing of sensory information. During this portion of the SWM timeline, various critical structures are constructing their representations; the HC, mPFC, and Re are tuning space-time representations to the current features. Once those representations are constructed, and perhaps even during the construction process, these representations must be compared against representations tuned during previous experiences in the environment. The final step of this context-sampling phase is the selection of a rule for how to respond to the current features of the environment. One has to make decisions about a plan for the course of action most likely to result in success. In the Delayed Non-Match to Position (DNMP) task, this portion of the timeline constitutes the first traversal (see Section 2.1.3 for more details).
In SWM, there is a delay period that links two segments of the task. The delay portion of the timeline requires retention of the contextual information that was just sampled in the first segment. The information most recently sampled must be useable during the next portion of the SWM timeline. In the DNMP task, this is accomplished through a forced delay period, during which the animal is confined.

The final portion of the SWM timeline is the representation-application segment. It follows a very similar pattern to the context-sampling segment, except that now the most critical information has already been sampled. The animal is again occupying space and time, with sensory information being used to construct a current representation of the environment and contextual. That information is again compared to previous representations, which now necessarily includes the immediately preceding context-sampling segment. A plan for how to behave was established during the previous context-sampling segment. That plan must now be either updated or reaffirmed, before it can be executed. Ultimately, the decision is made and the animal navigates to where they expect to find a reward. The final step is to update the general rule for how to behave during the task. Once they have arrived at the reward zone, their expectations are either confirmed or denied; they are either rewarded for a correct choice, or they are not. Evidence of SWR activity during these reward periods, in comparison to those events observed during sleep, suggest that these online consolidations have a stronger influence over successful SWM performance. Thus, the animal updates their rules and consolidates the additional experience. In the DNMP task, this entire third segment is contained within the second of the two paired maze traversals.
1.7 The Current Experiments

Decades of research has made it clear that each structure in the mPFC-Re-HC circuit is involved in some combination of spatial, temporal, or other memory-related processing. It is also clear that their interactions are both dynamic and critical to SWM and other memory-related cognition. Yet, critical questions remain. Can the encoding of information into neuronal representations for storage as rules be behaviorally distinguished from the neuronal processes involved in querying, updating, and applying those rule representations? When would each of these processes occur during a SWM task? How might the dynamics of the mPFC-Re-HC circuit support that? Are there distinctions between the contributions of the various input and output pathways? Do the components of SWM demand distinct directionalities to the flow of information? The contributions of the following experiments will be to add increased precision to the way we understand the timing of pathway-specific activity within this circuit to SWM processes. The current experiments will describe when during the above SWM timeline particular pathways within the mPFC-Re-HC circuit are necessary for accurate goal-directed navigation.
Chapter 2

METHODS: EXPERIMENTS 1 AND 2

2.1 Methods

All procedures and results reported below describe the work published in Maisson, et al. 2018 and submitted in Maisson, et al.

2.1.1 Subjects

Subjects were adult (at least 90 days old) male Long-Evans hooded rats that were ordered from Envigo (laboratory research supply company; Somerset, NJ) and weighed between 350 and 700 grams at the start of the experiments. Across all experiments, there were 12 control rats (6 Re-tdTomato, 3 dHC-tdTomato, and 3 mPFC-tdTomato) and 19 experimental rats (6 Re-ArchT, 7 dHC-ArchT, and 6 mPFC-ArchT). To encourage food-oriented foraging, rats were minimally food restricted to maintain 90% of their ad libitum body weight. Rats were grouped-housed until they began the study, after which they were individually housed in a temperature (70 – 74°F) and humidity (30 – 70%) controlled environment with a standard 12 hour light/dark cycle. Behavioral assays occurred during the light cycle. All procedures were carried out in accordance with the University of Delaware Institutional Animal Care and Use Committee and the NIH.

2.1.2 Apparatus

Behavioral assays were conducted on an elevated T-shaped maze (Figure 1). The maze was made of wood and painted white. The central stem measured 116 x 10 cm. Two reward arms extended 56.5 cm (with a width of 10 cm) in either direction from the T-junction. There were two return arms, each extending 112 cm (10 cm
wide) from the reward zone back to the start box. The walls of the maze were 6 cm high. Each reward arm was baited using weigh boats containing a chocolate sprinkle reward. The start box was positioned at the apex of the triangular maze, and served to confine the rats, during both the 20 second delay and the 40 second inter-trial interval (ITI), with a wooden barricade that was painted white. The maze was surrounded by a black curtain, containing distinct distal cues above each reward zone and at the T-junction. The room was dimly lit using two 1000 lm, 120 V, 60 Hz LED bulbs.
2.1.3 Behavioral Training

Prior to any surgeries or task exposure, animals were handled for a minimum of 5 days, for at least 10 minutes each day, to acclimate them to the handler. Following each handling session, rats were given chocolate sprinkles in their home cage, to habituate them to consuming the reward bait. After the handling period, rats were exposed to the reward zones of the T-maze each day. They were enclosed in the baited reward zone for a maximum of 90 seconds, alternating 6 times between sides. Once the rat demonstrated that they would reliably consume the reward (eating the bait within 90 seconds on all 6 trials for 2 consecutive days), they were advanced to the forced-run training. To shape their running behavior, rats were given 12 trials each day, pseudo-randomly alternating between reward zones, during which they were trained to run directly up the stem, turn to the reward zone, consume the reward, and return to the start box without stopping or turning around. When the rats demonstrated that they were able to meet these behavioral criteria on 10 of the 12 trials, for two consecutive days, rats were given the first of two surgeries, described in section 2.2.4. Following recovery, they started training in the delayed non-match to position (DNMP) task.

A single DNMP task trial consisted of two uni-directional runs through the environment, paired across a delay period. Before the start of each trial, both reward
zones were baited with 1-2 chocolate sprinkles. During the first traversal, a barricade blocked one of the two reward arms, forcing a given trajectory on the cue-guided, sample traversal. After consuming the reward, the rat returned to the start box. He is confined to this area with a wooden barricade for a 20 second delay period, during which time the barricade that was blocking one of the reward arms is removed. After 20 seconds, the rat runs the central stem again. As the barricade was removed, the rat was free to traverse either reward arm, only being rewarded for alternating relative to the previous traversal; thus, this is considered the memory-guided, choice traversal. On each day of training the rat runs 24 such trials, with pseudo-random alternation between forced turns. The rat remains in training until he is able to perform with at least 80% choice accuracy on 2 consecutive days, after which they are subjected to their second surgery, as described in section 2.1.4. Following a recovery period, that rat is retrained to pre-surgical choice accuracy before the experimental sessions begin, as described in section 2.1.5.

2.1.4 Surgical Procedures

The first of 2 surgeries was conducted after the animal’s running behavior was shaped and prior to training on the DNMP task. The purpose of this surgery was to deliver the optogenetic viral vector. The second surgery was conducted to implant an optical fiber. Two surgeries, rather than one, were done to reduce the probability of an implanted fiber dislodging from old acrylic and falling out prior to behavioral testing. During each surgery, rats were anesthetized with isoflurane at a concentration of 3.5-4%. During the surgeries, the rate of isoflurane was gradually reduced, preventing death by overdose. Atropine was also administered (.09 mg/kg), to reduce bronchial secretions. Prior to making an incision in the scalp, a local analgesia of diluted
lidocaine (2.5%, concentration of .2 mg/kg) was given. A single anterior-posterior (AP) incision was made to expose the skulls, which was then leveled. At least 30 minutes before the end of the surgery, banamine was injected as a general analgesic to aid in the recovery at a concentration of .05 mg/kg.

For the first study, investigating the role of Re local activity in SWM (Exp. 1), a craniotomy was made 2-2.5 mm posterior to bregma and 2 mm lateral to the midline. Two injections of a viral vector were delivered using a Hamilton syringe, at a lateral angle of 15° to the midline, to target the midline nucleus reuniens. Injections were controlled by a Pump 11 Elite Nanomite pump and Nanomite Injector Unit (Harvard Apparatus; Catalog# 70-4507, Serial# D-301251). The first of the 2 injections was delivered 2 mm posterior to bregma, 2 mm lateral to the midline, and 7.2 mm ventral to dura. The second injection was delivered 2.5 mm posterior to bregma, 2 mm lateral to the midline, and 7.4 mm ventral to dura. Vectors (ordered from the Boyden Lab stock, UNC Vector Core, titer < 10^{12} vg/ml) of either the control (AAV5-CAG-tdTomato) or opsion-positive (AAV5-CAG-ArchT-tdTomato) virus were delivered with a total volume of 1 µl (0.05 µl per site), at a rate of 0.1 µl/min. The promoter used in this vector, CAG, is a pan-neuronal targeting promoter. As a result, the vector is not selective to targeting any particular type of neuron. Thus, the resulting groups were identified as either Re-tdTomato or Re-ArchT. After the syringe was lowered, but prior to the start of the injection, the surround tissue was allowed to settle for 2 minutes. Then, following a 5 min injection, the syringe remained in place for another 5 minutes to allow the virus to diffuse out of the needle. After the syringe was removed following the final injection, and the craniotomy was sealed with GLUture, a quick sealing liquid for closing wounds, the incision was sutured closed. The rat was
removed from isoflurane and placed in a clean cage, on top of a heating pad until they could freely move about their home cage. The rats were then monitored for 5 days as they recovery.

For the study investigating the role of HC-Re (Exp. 2.1) and mPFC-Re (Exp. 2.2) input pathways in SWM, a similar injection procedure was followed, using all of the same equipment and viral vectors, but targeting different structures. For Exp. 2.1, four craniotomies were made; two above each hemisphere. On each side of the midline, a craniotomy and injection targeted the dHC at 5.3 mm posterior to bregma, 1.2 mm lateral to the midline, with the injection at a depth of 3.2 mm ventral to dura. A second set of craniotomies and injections targeted dHC at 5 mm posterior to bregma, 3.2 mm lateral to the midline, with the injection from the needle tip held at a depth of 2.5 mm ventral to dura. These injections thus constituted the *dHC-tdTomato* and *dHC-ArchT* groups.

For Exp. 2.2, a single, large craniotomy (1 mm diameter) was made above the injection site for four injections targeting the mPFC. The same injection coordinates were used within each hemisphere. Both injections were delivered to a site that was 3.2 mm anterior to bregma and .5 mm lateral to the midline. However, each of the injections (.05 µl) was delivered at a different depth. The first was administered at 4.6 mm ventral to dura. After waiting the standard 5 minutes for the virus to diffuse, the needle was then raised to a depth of 2.7 mm ventral to dura, and the same injection procedure was followed at the new depth.

Precisely the same anesthesia, analgesia, incision, and recovery procedures were followed for the later fiber implant surgery. After the incision was reopened, and the skull leveled, bone screws were drilled around the site of the fiber implant, to act
as anchors for the dental acrylic. For Exp. 1, the previously sealed craniotomy was reopened. For Exp. 2, an additional craniotomy was performed above the site of the fiber implant. An optical fiber was implanted at a lateral angle of 15° to the midline; 2.3 mm posterior to bregma, 2 mm lateral to the midline, and 7 mm ventral to dura. GLUture was used to fill the craniotomy and partly stabilize the fiber implant, as the substance tends to harden upon contact with tissue. Prior to performing the implant surgeries, the ceramic ferrules of the fibers were scored to provide a rough surface to which the acrylic could bind. After the implant, dental acrylic was applied adhering the scored fiber ferrule to the bone screws, and the incision was then resealed.

2.1.5 Behavioral Testing

Following recovery from the implant surgery and retraining to pre-surgical performance, rats started behavioral testing in the various experimental conditions. The exact same DNMP task procedure as described for the training (section 2.1.3) was applied to the behavioral testing. Behavioral testing procedures were executed on a setup configured by Plexon, Inc., Dallas, TX. The rat’s location was monitored with their CineLab video tracking software, and their PlexBright Optogenetic Stimulation System relied on their Radiant v2 software to activate a compact LED module, radiating green light (525 nm) through a patch cable with a 200 µm diameter core and a numerical aperture of .66. For Exp. 1, four experimental conditions, each conducted on a separate day, were defined by which portion of the task the rat was in when the light was delivered; entire trial, sample traversal, delay period, and choice traversal (Figure 2). There were 4 alternating testing blocks (light-off and light-on), each consisting of 6 trials. Exp. 2 was conducted as a follow-up to the results reported in Exp. 1, showing selective contributions of the Re to a particular traversal. As such, the
entire trial experimental condition was not conducted for Exp. 2, which focused only on the individual traversals.

Figure 2. *Schematic of Experimental Conditions.* Modified from the original in Maisson et al, 2018. The green area indicates the portion of the maze included during the light-on manipulations. The orange dot indicates the baited reward zone. The black bars indicate barricades that either force a trajectory or confine the rat. The *left* panel indicates the sample traversal experimental condition, showing that the light was on during the stem and reward arm, turning off upon entering the return arm. The *middle* panel indicates the delay period experimental condition, as the light was on only while the rat was confined to the start box during the 20 second delay. The *left* panel indicated the choice traversal experimental condition, showing the barriers had been removed and the light was on during stem and reward arm occupancy.

### 2.1.6 Histology

After being tested in each of the 4 experimental conditions, rats were anesthetized with isoflurane and given an overdose of sodium pentobarbital (Euthasol,
.5 ml), to stop the contractions of the diaphragm but leave the cardiac rhythm intact. When breathing ceased, the rat was transcardially perfused with 200 ml of tris-buffered saline (TBS, pH 7.2 – 7.4), followed by 200 ml of 4% paraformaldehyde (PFA, pH 7.2 – 7.4).

After extraction, and post-fixation for at least 24 hours in 4% PFA, the brain was transferred to a 30% sucrose solution until it sunk. Brain sections (40 µm thick) were mounted to microscope slides with Prolong Diamond mounting medium (with DAPI). The slides were washed with TBS, and sealed with a coverslip. Images were collected using a Zeiss 880 confocal microscope, with a C-Apochromat 10x/0.45 W lens, to confirm fiber tip location and virus expression; each was localized by comparing the images against The Rat Brain Atlas.

The ArchT protein is a proton pump that is only activated by light within a highly restrictive wavelength around the peak activation at 566 nm. Following the development of the opsin, investigators determined that light could not activate opsins further than 2 mm from the light source. Traveling through 2 mm of saline solution, light power is reduced to 10% of that at the source (Smith & Smith, 2014), which is insufficient to activate the opsin. Therefore, the principal histological determinant for including rats in the final set was localizing expression of the virus below the tip of the fiber and within the activation range of the light (3.53 mm³), as determined by the formula for the volume of a symmetrical right-circular cone, with a height and radius of 1.5 mm as emitted by optic fibers with a .66 numerical apertures.

\[ V = \pi r^2 \frac{h}{3} \]

Based on The Rat Brain Atlas, the rat mPFC extends approximately 2.64 mm, the Re approximately 2.4 mm, and the HC approximately 4.68 mm rostro-caudally. To
determine if viral spread was sufficient to encompass the target structure, coronal sections between ± 3 mm from the most anterior to the most posterior injection site were collected for the structures of interest in the given experiment. The Re, dHC and mPFC, depending on the experiment, were consistently imaged to gauge viral expression.

2.1.7 Statistical Analysis

Data were analyzed using a 2 (light) x 2 (group) mixed-measures ANOVA, comparing the performance of Re-tdTomato and Re-ArchT choice accuracy during light-off and light-on trials in each experimental condition. Rats in one study spontaneously alternated at approximately 67%, without training, allowing investigators to identify this rate as "true chance" performance. One of our early objectives for Exp. 1 was to determine the magnitude of performance impairment, relative to variability within the control group, which we could use to control for variability in performance due simply to daily performance variability in behavior. Based on the data from Exp. 1, and in light of the previous study showing a "true chance" for alternation performance, data from individual testing sessions were excluded from the final set if choice accuracy across all light off trials was below 67%; if control trial performance on a given day was below chance. Additionally, in Exp. 1, to confirm that choice accuracy was returning to baseline after each light-on trial, a 2 (group) x 4 (light-block) ANOVA was conducted. As discussed below, there were no significant findings, so this analysis was not repeated for Exp. 2. Post-hoc pairwise comparisons were conducted, using Bonferroni’s correction, to further investigate significant interactions. For behavioral analyses in Exp. 2, the 3 control
groups were collapsed into a single \textit{tdTomato} group. Statistical tests, computed with SPSS, used an alpha level of < .05 to reach significance.
Chapter 3

EXPERIMENT 1: CONTRIBUTIONS OF REUNIENS ACTIVITY TO SPATIAL WORKING MEMORY

3.1 Introduction

Over the course of several decades, the roles of both the mPFC\textsuperscript{70,71,79,80,82,84,94--96} and the HC\textsuperscript{30,36,97--101} in various cognitive functions, such as memory and decision-making, have been thoroughly probed. Complex functions, such as working memory demands applied to a task requiring spatial navigation, have also been associated with the interactions between the mPFC and the HC\textsuperscript{74,77,86,102--108}. However, increasing attention is being given to the unique contributions of the structures that mediate their activity and interactions during behavior. In particular, investigations into the organization, connectivity, and neuronal activity patterns of the Re have generated compelling evidence of its criticality to SWM\textsuperscript{18,83--85,87--90,109,110}.

Previous studies have shown that, while the HC does project directly to the mPFC\textsuperscript{22,111,112}, no such direct reciprocal projections are known to exist. Additionally, the only direct projections from the mPFC to the HC yet identified are in mice, and are sparse at best\textsuperscript{23}; no such projections have yet been reported in rats, suggesting that this may not be a generalized phenomenon. The most direct pathway for bi-directional communication between the mPFC and the dHC appears to be the di-synaptic route through the Re\textsuperscript{15--17,22,24,25}. In fact, some mPFC neurons synapse on Re neurons that then project to the dHC\textsuperscript{17}. Furthermore, a small population of neurons in the Re is known to simultaneously project to the mPFC and the dHC\textsuperscript{25}. As such, the Re appears to be anatomically well positioned to orchestrate interactions between the mPFC and dHC.
There have been several recent reports of investigations into the necessity of the Re to SWM. Suppressing the Re, by infusing a GABA$_a$ receptor agonist (muscimol), impaired accurate alternation during the delayed alternation task$^{90}$. It was also reported that several measures of mPFC-dHC synchrony, first shown to be heightened during undisturbed behavior, were significantly reduced following the Re muscimol infusion. This study showed that not only was the Re necessary for this SWM task, but that the activity of the Re influences mPFC-dHC interactions in a way that appears to support successful SWM.

While muscimol has been a powerful tool in probing the link between activity within the brain and observed behaviors, it is limited in both its temporal precision and its selectivity for neuronal populations. It indiscriminately inhibits any cells with GABA$_a$ receptors within range of the infusion spread for up to 30 minutes. Additionally, the delayed alternation task consists of two traversals during which the rat is free to choose their trajectory, prohibiting a clear separation between the cognitive demands of each traversal. Similarly, both traversals of the DNMP task likely require neuronal activity to encode a representation of the environment and integrate it with representations of relevance to goal-directed navigation. By contrast, the decision-making component of SWM is restricted to the second traversal in the DNMP task. Selectively interfering with this activity would require high fidelity temporal control, as can be offered by optogenetics. Opsins have been shown to activate within ~10 ms of delivering light within the requisite range of wavelengths, and deactivate equally rapidly when the light is turned off$^{113,114}$.

By combining the temporal precision of optogenetic technology with the behavioral segmentation offered by the DNMP task, the current experiment will
significantly expand our understanding of the necessity of Re to SWM. Particularly, this experiment will be the first to investigate contributions of the Re during isolated components of working memory.

3.2 Results

First, we confirmed that the rats were expressing virus in the Re, with the optical fiber tip appropriately located above the nucleus (Figure 3). All 12 rats were able to be included in the study. Six rats were included in the Re-ArchT group and 6 in the Re-tdTomato group. In all cases, virus was confirmed to be present within the ventral midline thalamus. There was an average lateral spread of .95 mm to either side of the midline ($SD = .39$), and an approximated anterior-posterior spread of 2.74 mm ($SD = .12$; range: 2.52 – 3.08 mm). Fiber tips were always confirmed to be dorsal to the expression of the virus.
Figure 3. Exp. 1: Histology. Modified from the original in Maison et al, 2018. A) A representative image of viral expression in the Re (red fluorescence) and the fiber implant (negative space left by the angularly implanted fiber). The inset panel displays an image of the same slide at increased magnification above the expression site. B) Modifications of plates from The Rat Brain Atlas, showing the location of the Re. The maximal (light red shading) and minimal (dark red shading) spread imaged during histology are over-laid onto their approximate locations relative to the plates. The black dots in the middle panel indicate the approximated location of the fiber tips.

We analyzed choice accuracy during the entire trial experimental condition, to first replicate previous findings showing disrupted SWM performance with generalized Re suppression via muscimol infusion. Indeed, suppressing the Re during the entirety of the DNMP task was associated with disrupted choice accuracy (Figure 4A). The ANOVA revealed a significant light X group interaction (F(1,10) = 8.67, p = .015). Two-tailed, simple main effects t-tests showed that the Re-ArchT group performed significantly worse during the light-on trials (Mean (M) = 61.8%, SD = 7.65) than during the light-off trials (M = 84.7%, SD = 7.76; t(5) = 5.7, p = .002). By contrast, the Re-tdTomato group performed similarly well under both light conditions (light-off: M = 84.7%, SD = 6.27; light-on: M = 81.9%, SD = 16.17; t(5) = .5, p = .638). These results corroborate the findings of previous studies, showing that the Re is necessary for supporting SWM.

We next wanted to take advantage of the temporally precise control offered by optogenetic techniques, by probing the individual components of SWM. To do so, we introduced the light, ostensibly depolarizing opsin-expressing Re neurons, during only the cue-guided traversal, theoretically disrupting the encoding of task-relevant information. The ANOVA revealed a significant light X group interaction (Figure 4B)
in this sample traversal experimental condition \((F(1,10) = 6.013, p = .034)\). Two-tailed simple main effects t-tests showed that the Re-ArchT group had significantly reduced choice accuracy during trials on which the light was on for the sample traversal only (\(M = 65.3\%, \ SD = 18.38\)), as compared to when the light was off (\(M = 82.6\%, \ SD = 5.54\); \(t(5) = 2.83, p = .036\)). However, the Re-tdTomato group performed at similarly high choice accuracy for both light conditions (light-off: \(M = 81.9\%, \ SD = 12.27\); light-on: \(M = 82.6\%, \ SD = 10.68\); \(t(5) = -.17, p = .872\)).

We also administered the light exclusively during the delay period (Figure 4C), and during the memory-guided, choice traversal (Figure 4D). No significant interactions were discovered by the ANOVAs for either the delay period \((F(1,10) = .029, p = .868)\) or choice traversal \((F(1,10) = .534, p = .482)\) experimental conditions. Furthermore, there were no detectable main effects for either light \((F(1,10) = 1.416, p = .262)\) or group \((F(1,10) = .546, p = .477)\) in the delay period experimental condition. In the choice traversal experimental condition, no significant main effects for either light \((F(1,10) = 1.275, p = .285)\) or group \((F(1,10) = 4.310, p = .065)\) were observed. These results suggest that suppression of local neuronal activity within the Re is selectively associated with disruptions to processes supporting the encoding of task-relevant information.

Finally, we wanted to confirm that choice accuracy would remain consistent across testing sessions. This would confirm that any disruptions of performance were restricted to when the Re was actively being suppressed, indicating that SWM processes, and not learning or reconsolidation, were being disrupted. A set of ANOVAs confirmed that, in fact, the suppression of the Re was associated with SWM deficits. During the entire trial experimental condition, no group X block interaction
(F(1,10) = 2.902, p = .119) was detected. The same was true during the sample traversal (interaction: F(1,10) = 1.369, p = .21; block: F(1,10) = 1.596, p = .21; group: F(1,10) = 1.982, p = .19), delay period (interaction: F(1,10) = .53, p = .665; block: F(1,10) = .417, p = .742; group: F(1,10) = .546, p = .477), and choice traversal (interaction: F(1,10) = .943, p = .432; block: F(1,10) = .885, p = .46; group: F(1,10) = 4.310, p = .065) experimental conditions. These results suggest that, in fact, the manipulation of neuronal activity was selective to instances when the light was being delivered through the patch cable.
Figure 4. Behavioral Effects of Suppressing Re Excitatory Activity. Modified from the original in Maisson et al, 2018. Grey-colored lines indicate choice accuracy for the Re-ttdTomato group during light-off and light-on trials. Green-colored lines indicate choice accuracy for the Re-ArchT group during light-off and light-on trials. Error bars show the standard error of the mean. The asterisk denotes statistically significant differences. The figure shows that only choice accuracy during the entire trial (upper left panel) and sample traversal (upper right panel) experimental conditions was impaired.

3.3 Discussion

Based on previous findings implicating the Re as a critical structure in supporting SWM, we expected to find that optogenetically suppressing the Re during the entirety of the DNMP task would impair choice accuracy. In fact, the results of the current study support this conclusion. The current study expanded on what is known about the role of the Re by demonstrating that the perturbation of the nucleus is only associated with impaired SWM task performance when the disruption occurs during the cue-guided, sample traversal.

In the current study, optogenetic suppression was achieved using a light-activated proton pump, the production of which was coded for by a viral vector that depended on a pan-neuronal promoter to identify target cell-types. To date, there are no known populations of inhibitory neurons within the Re. This would suggest that the effects of delivering light directly to the Re, where the ArchT was expressed, had a depolarizing effect on local excitatory activity within the nucleus. This mechanism effectively simulates, on the millisecond time scale, the effects of muscimol; inhibiting local excitatory activity by binding with GABAa receptors. We can reason that, after extensive training on the alternation-based DNMP task, rats are applying the memory of contextual information encoded during the forced traversal to
the choice traversal to guide their behavior and navigate to the alternate arm. Thus, the results of the current study demonstrate that excitatory activity within the Re is critical during the forced traversal to support these processes.

However, the network mechanisms and timing dynamics for when Re local excitatory activity is necessary is still not fully clear. There are several reasonable explanations for how Re suppression may affect the circuit. Firstly, excitatory activity within the Re may support a simple relay of signaling, analogous to other thalamic relay nuclei, between the mPFC and HC that is necessary during the forced traversal, such that suppressing Re excitatory activity prevents bi-directional outputs from transmitting across this di-synaptic route. Secondly, in addition to receiving excitatory input from structures critical to SWM, such as mPFC and HC, the Re also receives inhibitory and modulatory inputs from other structures. It is possible that the essential role of local Re excitatory activity is to incorporate these multiple inputs and act as a form of pacemaker, orchestrating the timing for mechanisms of mPFC-HC synchrony. Given that several mechanisms of mPFC-HC synchrony have been shown to be critical for SWM task performance, it is possible that the role of Re excitatory activity in orchestrating synchrony is selective to the identification and integration of task-relevant, contextual information for guiding navigation.

Additionally, it is possible that local computations within the Re itself might be necessary for processing, integrating, and associating streams of information for successful SWM performance. Previous work has characterized neuronal activity within the Re suggestive of processing spatial information, such as neurons that have tuning curves for specific orientations of the head in space. It is possible that excitatory neurons within the Re may engage in computations that are distinct from
those offered by other structures within the network, namely the mPFC and the HC. Furthermore, it would be likely that these computational processes within the Re are necessary during the forced traversal, such that suppressing local Re activity during this segment of the task impairs choice accuracy.

Aside from the specific mechanisms to which excitatory activity within the Re contributes, it is also unclear from the current study what, if any, directionality exists within the network. The results clearly demonstrate that Re excitatory neurons must remain undisturbed during the forced traversal, if the animal is to perform the DNMP task with highly accurate alternation between paired traversals. However, it is not yet clear whether this traversal-selective necessity is a function of generalized coordination across the circuit, or the activity of any single structure directing the activity of the others. Nonetheless, the current results offer insight into how the brain may yield complex behaviors, like SWM, and generates interesting questions about the activity of the circuit and compelling directions for future investigation.
Chapter 4

EXPERIMENT 2: IDENTIFYING THE CONTRIBUTIONS OF INPUT PATHWAYS TO THE NUCLEUS REUNIENS

4.1 Introduction

The necessity of the HC to spatial navigation and episodic memory has been firmly established. The mechanisms and patterns by which neuronal activity patterns within the HC represent space\(^{120-124}\), time\(^{125-129}\), and space-time episodes\(^{130,131}\) is a subject of on-going and concerted investigation. The mPFC has been heavily implicated in a wide range of behaviors, including executive commands, decision-making, and memory consolidation\(^{42,70,71,79,80,82,84,94-96,108,132}\).

It is clear that both the mPFC and HC are involved in several critical, overlapping functions, such as working memory. This led researchers to reason, and then heavily investigate, that the two structures interact during these behaviors. Evidence indicates heightened interactions between the two, during behaviors like SWM\(^{77,78,81,90}\). In fact, mPFC-HC interactions are particularly critical when the working memory demand is high\(^{87,89}\), as in a task with a long delay period. The extent to which LFP oscillations in the 7-12Hz (theta) range in both the mPFC and HC fluctuate synchronously is greater at a maze decision point when the task requires working memory as compared to when it simply requires cue-based navigation; HC oscillations in this frequency band appear to be leading those in the mPFC. Additionally, higher frequency gamma oscillations (30-80Hz) in the mPFC are also more synchronous with HC theta during a task that demands working memory, though mPFC gamma appears to be leading HC gamma instead of theta. Synchrony alone might suggest that the two structures are anatomically linked, as periodic fluctuations in excitability in one structure must have a way of phase-locking with that of another.
If we recognize a statistically significant lead index in synchrony as evidence of directionality, we could reason that the two structures are likely linked fairly directly. That the lead indices are bi-directional, depending on the frequency band in question, suggests that the anatomical links are likely also bi-directional. The most direct route of bi-directional signaling appears to be through the Re\textsuperscript{15–17,22,25}. Each structure sends excitatory projections, from along their dorso-ventral extents, which synapse on excitatory neurons within the Re.

One study reported that silencing the Re decreases traces of mPFC-HC synchrony at seemingly critical junctures during behavior\textsuperscript{90}. The study further reported that the perturbed circuit dynamics were associated with an impairment to accurate navigation in a delayed alternation task. As described in chapter 3, we followed up on these findings by refining the manipulation of Re activity in a temporally precise way. The results indicated that it was particularly important for the activity of the Re to remain undisturbed during the forced traversal, during which contextual information that would inform navigation on the immediately subsequent traversal must be encoded.

To probe more deeply into the activity across this SWM-supportive circuit, we decided to suppress individual pathways, rather than whole structures. Given the role of the dHC in representing space-time episodic information, it would be neither surprising nor novel to report that suppression of the entire dHC disrupted performance during a spatially-dependent working memory task. However, far fewer studies have reported on the output of the dHC to downstream structures. If the output of information from the either the mPFC or the dHC to the Re was essential to either the encoding of current context and/or the enrichment of that information with
representations of relevance to goal-directed navigation, then suppressing the dHC-Re and/or mPFC-Re pathways during the forced traversal would impair choice accuracy. If either structure was necessarily involved in integrating the conjunctive representations from the forced traversal with the current context, and/or responsible for transmitting command information for how to apply these representation to a navigational decision, then disrupting either pathway during the choice traversal would impair accurate alternation.

To examine the behavioral relevance of the mPFC- and dHC-Re pathways to the timeline of SWM components, we combined the anatomical and temporal specificity of optogenetics with the behavioral compartmentalization offered by the DNMP task. Projection terminals from either the dHC of the mPFC were modified to rapidly deactivate on-demand. However, by implanting an optical fiber at the target site of those neurons’ projections, only their terminals were hyperpolarized and their synaptic communication suppressed. Thus, highly selective pathways were perturbed during distinct phases of the DNMP task to, for the first time, elucidate directionally-specific contributions, along select anatomical pathways, during particular moments of the SWM timeline.

4.2 Results

As previously describe in chapter 3, the first step was to determine that the virus was sufficiently expressed, both at the injection site and at their projections to the Re. The average spread of virus expression was estimated at each site and the projections to the Re were determined to be within range of the implanted fiber (Figure 5). Across the mPFC-tdTomato and mPFC-ArchT groups, the mean radius of the medio-lateral spread within the mPFC was 1.39 mm (SD = .51) from the midline.
The mean dorso-ventral spread was 3.07 mm (SD = .81). Across the *dHC-tdTomato* and *dHC-ArchT* groups, the mean radius of the medio-lateral spread within the dHC subregion was 3.29 mm (SD = 1.36), centered on the midline. The mean dorso-ventral spread was .9 mm (SD = .56) at any given medio-lateral position along the septo-temporal axis.
Thus, based on histological and behavioral criteria, there were 12 rats included in the *tdTomato* group in all 3 experimental conditions. Two *dHC-ArchT* rats were unable to complete all 3 testing days, because their fiber implants broke prematurely. There were 7 *dHC-ArchT* rats and 6 *mPFC-ArchT* in the sample traversal experimental condition. For both the delay period and choice traversal experimental conditions, there were 6 *dHC-ArchT* rats and 6 *mPFC-ArchT* rats in the final set.

### 4.2.1 Hippocampal Input to the Nucleus Reuniens Supports Encoding

Based on the findings reported in Exp. 1, namely that Re suppression during the sample traversal was associated with choice accuracy impairments, we were able to conclude that optogenetic silencing of local Re activity only impaired choice accuracy when the manipulation occurred during the cue-guided, forced sample traversal. We wanted to determine if perturbing the pathway specifically from the dHC to the Re during the sample traversal would similarly impair choice accuracy, and/or if suppression of this pathway at any other point during the SWM timeline would disrupt performance. Choice accuracy for the sample traversal experimental condition, in
which light was administered only during the cue-guided run, was analyzed with a 2 x 2 mixed-measures ANOVA (Figure 6A). The test showed a significant light X group interaction (F(1,15) = 9.302, p = .008). A two-tailed paired t-test to compare simple main effects showed that the dHC-ArchT group choice-accuracy was significantly impaired (t(6) = 3.198, p = .019) when the light was on (M = 69.05%, SD = 11.501) during the sample run, as compared to when the light was off (M = 86.9%, SD = 6.56). By contrast, the tdTomato group performed similarly well with both light off (M = 84.2%, SD = 8.29) and light on (M = 83.8%, SD = 8.44).

Further corroborating the conclusions from Exp. 1, there were no performance deficits associated with suppressing the dHC-Re pathway in either the delay period (F(1,15) = .034, p = .857) or the choice traversal (F(1,15) = .057, p = .814) experimental conditions (Figure 6A). However, in the delay period experimental condition, there was a significant main effect of light (F(1,15) = 4.992, p = .041) that was not detected in Exp. 1. While this test did not reach significance in Exp. 1, there was a minor numerical decrease in choice-accuracy during the light-on trials, suggesting in conjunction with the current results the possibility of a minor distractor effect of the light. This could be explained by the white-painted barricade, as the light was bright enough to reflect off the surface during the delay-period. It is possible that the bright reflection of the light on the white barricade was distracting to the animals, regardless of virus type, drawing their attention away from the salient information used to inform their decision during the subsequent traversal.
Sample  Delay  Choice

A  
dHC-to-Re

B  
mPFC-to-Re
Figure 6. *Behavioral Effects of Suppressing mPFC-Re and dHC-Re Input Pathways.* Black-colored lines indicate choice accuracy for the *tdTomato* group during light-off and light-on trials. Green-colored lines indicate choice accuracy for the respective *ArchT* groups during light-off and light-on trials. Error bars show the standard error of the mean. An asterisk above a black bar denotes a statistically significant main effect. Lone asterisks identify means that are statistically significantly different from the other groups. A) Shows the choice accuracy for the *dHC-ArchT* group during the sample traversal (*left panel*), delay period (*middle panel*), and choice traversal (*right panel*) experimental conditions. B) Shows the choice accuracy for the *mPFC-ArchT* group during the sample traversal (*left panel*), delay period (*middle panel*), and choice traversal (*right panel*) experimental conditions.

4.2.2 Prefrontal Input to the Nucleus Reuniens Supports Retrieval

Next, we investigated the contributions of the mPFC-Re input pathway in SWM (Figure 6B). mPFC-Re terminals were suppressed during either the sample traversal, delay period, or choice traversal, and choice accuracy on the choice traversal was recorded. A 2 x 2 mixed-measures ANOVA, testing choice accuracy during the sample traversal experimental condition, showed a significant light X group interaction (F(1,14) = 6.379, \(p = .024\)). A two-tailed paired t-test to compare simple main effects showed that the *mPFC-ArchT* group choice-accuracy was significantly impaired (t(5) = 4, \(p = .0103\)) when the light was on (M = 73.6\%, SD = 9.74) during the sample run, as compared to when the light was off (M = 84.7\%, SD = 8.19). By contrast, the *tdTomato* group performed similarly well with both light off (M = 84.2\%, SD = 8.29) and light on (M = 83.8\%, SD = 8.44). Further corroborating the conclusions from Exp. 1, there were no performance deficits associated with suppressing the mPFC-Re pathway in the delay period (F(1,15) = .747, \(p = .401\)). Again, there was a significant main effect of light (F(1,15) = 5.956, \(p = .027\)), further suggesting the likelihood of a distractor effect.
Interestingly, there was clear evidence that the mPFC-Re pathway is necessary during the memory-guided, choice traversal. Strikingly, only the mPFC-Re pathway, relative to either the Re itself or the dHC-Re pathway, appears necessary during the choice traversal to support SWM performance. The 2 x 2 mixed-measures ANOVA revealed a significant light X group interaction during the choice traversal experimental condition as well (F(1,16) = 5.953, p = .027). A two-tailed paired t-test of simple main effects (t(5) = 5, p = .004) showed that the mPFC-ArchT group’s choice accuracy was significantly impaired during light-on trials (M = 68.1%, SD – 9.74), as compared to light off trials (M = 88.9%, SD – 6.8). The tdTomato group performed similarly well under both conditions (light-off: M = 85.1%, SD – 9.64; light-on: M = 80.6%, SD – 12.98)

### 4.3 Discussion

The current experiment extends findings on the criticality of the mPFC and the HC in supporting SWM. When manipulations of the circuit were restricted to silencing Re activity, the results demonstrated that suppressing excitatory activity within the Re during the forced traversal impaired choice accuracy on the DNMP task. The current experiment builds on these results by demonstrating that there are distinctions between the contributions of inputs from the dHC and the mPFC to the Re during the SWM pipeline. Similarly, suppressing the dHC-Re and mPFC-Re pathways during the forced traversal each impaired choice accuracy. Interestingly, the results of the current study also indicated that suppressing the mPFC-Re pathway during the choice traversal was sufficient to disrupt choice accuracy.

The same virus was used in the current experiment as was used to suppress Re activity, relying on a pan-neuronal promoter to target any neurons within either the
dHC or the mPFC. Each of these structures is known to be home to inhibitory neurons\textsuperscript{133,134}, which were likely also transfected by the virus. However, as yet, there is no evidence of long-rang inhibitory projections from either structure to the Re. The fiber was implanted above the Re, thus restricting the range of the light to envelope this nucleus, and perhaps some portion of the adjacent rhomboid nucleus. Given that the sites of the injections were relatively remote and the projections from each structure to the midline thalamus have highly selective targets, the only ArchT proteins within activation range of the light would be located at the axon terminals of excitatory projections\textsuperscript{93,115}. Thus, activating the ArchT must have suppressed excitatory-excitatory signaling from either the dHC or the mPFC to the Re by hyperpolarizing the terminals and reducing the probability of synaptic transmission.

Suppressing excitatory-excitatory signaling along either the dHC-Re or mPFC-Re pathways during the forced traversal is sufficient to impair accurate alternation during the DNMP task. Additionally, perturbing only the mPFC-Re pathway during the choice traversal is sufficient to impair accurate alternation. Suppressing the Re alone was insufficient to identify whether there were directionally specific streams of information during components of SWM, or distinctions between parts of a SWM task during which the entire circuit is required to cooperate in support of the behavior. Again, we can reason that rats have been trained to reliably link two paired traversals across a delay period. The maze environment during the sample traversal includes a barricade that forces a behavior which then informs the free navigation during the choice traversal. In all experiments, suppressed activity was restricted to excitatory neurons, due to the fact that the mPFC and dHC only send excitatory projections to the Re and that the Re is not known to host any inhibitory neurons. Therefore, excitatory-
excitatory signaling along the mPFC-Re and dHC-Re pathways and excitatory signaling within the Re during the sample traversal are necessary for choice accuracy. Thus, it is likely that there is system-wide excitatory activity that supports the SWM processes required during that forced traversal. During the choice traversal, by contrast, there is a highly specific directionality to the flow of information from the mPFC.

It is possible that both the mPFC and dHC engage in neuronal computations during the forced traversal, the output of which must be able to be transmitted freely and bi-directionally through the Re during this portion of the task. While the Re does offer the most direct route for bi-directional communication between the mPFC and dHC, it is not the only known pathway. This would suggest that the activity of the Re, in particular, is engaged in unique computations necessary to SWM rather than a simple patch-through of excitatory signaling between the mPFC and dHC. The activity of each structure, as well as the exchanges between them, must support the representation of the current environment and the enrichment of that representation with goal-directed utility.

What is most compelling about the results of the current study is that, from among the manipulated signals, only the excitatory input from the mPFC appears to be necessary during the choice traversal. This clearly demonstrates that the circuit is treating the two traversals differently, perhaps suggesting that activity during one traversal supports information processing that is distinct from that of the other traversal. Excitatory mPFC input to an otherwise undisturbed Re, likely influences Re computations in a very particular way. This influence must play a particularly critical role during the choice traversal, such that suppressing the mPFC-Re pathway during
the choice phase impairs choice accuracy. By contrast, suppressing all local Re
activity is not selective enough to yield similar impairments.

As behavior was the only measured variable, the results of the current
experiment cannot offer clear conclusions about the exact affected processes.
However, it is likely that the cognitive demands of individual components of SWM,
and the respective neuronal processes, are distinguishable. As such results are only
possible given the combination of separating phases of a SWM task and the
temporally precise control offered by optogenetics, the current study significantly
expands our understanding of working memory, and the necessary mPFC-Re-dHC
circuit dynamics.
Chapter 5
SUMMARIZING THE ROLE OF THE PFC-RE-HC CIRCUIT DURING SPATIAL WORKING MEMORY

5.1 Components and Timeline of Spatial Working Memory

To recapitulate, we have broken down SWM into its spatial, temporal, and stored information components. Each of the three components of SWM can be represented by activities across our mPFC-Re-HC tri-regional circuit. Given the discovery of time cells in the HC, some have argued that neuroscience would be remiss not to join physics in its conclusions about space-time\textsuperscript{128}; It should not be surprising to find overlap between space and time representations. If we consider that time is a construct by which factors are sequenced\textsuperscript{135}, and space is marked by its utility in representing contexts\textsuperscript{11}, then memory should be a representation of sequenced contexts; time would be the mechanism by which episodes within a given spatial or non-spatial context are sequenced\textsuperscript{128}, which is ultimately a memory. Representations of space and time are just how we, as observers of the activity, label them based on the tools we use to measure them; these are actually representations of episodes that occur within a space at a given time\textsuperscript{128,131,136,137}. Working memory, then, would refer to the process by which contexts are encoded into neuronal representation, sequenced, applied to decision-making processes, and executed\textsuperscript{124}.

Thus, the mechanisms by which the PFC-Re-HC circuit supports SWM can be organized into a logical SWM timeline. This timeline consists of “sampling the context”, “retaining the information”, and “applying the representation”. The context sampling portion, served by the sample traversal of the DNMP task, naturally requires that previous experiences with the task be recalled; part of the memory component of SWM. Active representations for contextual features must be constructed (i.e., space
and time). Current context is compared to previous experiences with the task-rules, and a plan of action is likely formed. Next in the SWM timeline, the delay period of the DNMP task constitutes the “information retention” portion. Finally, there is the representation application portion, constituted by the choice traversal of the DNMP task. The animal again moves along the path through the environment, constructing a representation of context; space and time components. These representations are compared to previous representations, from both the preceding traversal and from earlier experiences; the memory component of SWM. A decision is made, based on the available information, to navigate toward one of the available reward zones. Lastly, upon either receiving a reward for making a correct choice or not, the contextual information and memory for the rules of the task are reconsolidated and updated.

The current studies offer insight into several critical questions about how PFC-Re-HC circuit supports SWM. Can the encoding of information into neuronal representations of learned rules be behaviorally distinguished from the neuronal processes involved in utilizing those rule representations? When would each of these processes occur during a SWM task? How might the dynamics of the mPFC-Re-HC circuit support that? Are there distinctions between the contributions of the various input and output pathways? Do the components of SWM demand distinct directionalities to the flow of information?

5.2 Circuit-wide Contributions to Context Sampling

The results reported from experiment 1, in which the Re itself was optogenetically inhibited, demonstrate that the ventral midline thalamic nucleus distinctly contributes to the context sampling portion of SWM. Suppressing the Re
only produced performance deficits in the DNMP task during the sampling phase of the task. In particular, choice accuracy dropped to chance levels. Therefore, the effect of suppressing the Re during the entire task was likely driven by context sampling processes during the first traversal. In experiment 2, we extended these findings by demonstrating that disrupting both the mPFC-Re and the dHC-Re pathways during context sampling impaired choice accuracy. In another study\textsuperscript{78}, mPFC neurons distinguished between both upcoming trajectory and traversal on the DNMP task. When the vHC-mPFC pathway was optogenetically suppressed during the sample traversal, mPFC firing rates continued to distinguish between traversal but failed to accurately code for upcoming trajectory. Multiple studies of SWM, using the DNMP task, have aggregated to show system-wide context sampling processes.

Working through our delineated SWM timeline, the context sampling portion consists of memory for a previous experience, active representations of the current context, comparisons between these two streams of information, and the formulation of a rule-based action plan. It is clear from current and previous investigations of the PFC-Re-HC circuit during the DNMP task, that the context sampling portion of SWM requires the input of all three structures. If only the mPFC were required, disrupting the dHC-Re pathway would not cause a deficit. If only the HC were required, the same would be true of disrupting the mPFC-Re pathway. Thus, we can postulate about the role the structures and pathways play in each aspect of the context sampling portion of SWM.

Some argue that long-term storage of representations for previous experience are found within the neocortex\textsuperscript{40}. Furthermore, the PFC has often been shown to contribute heavily to executive control\textsuperscript{138}. In fact, even in reports of PFC
representations of spatial information there usually appears to be a necessary contingency for representing goal-orientation within the spatial code. We can reason that goal-directed intent is a function of understanding how one’s current location can be used to inform a decision. It follows, then, that goal-direction requires a representation of a previous experience in which location and direction led to a desired outcome, such as a reward. However, it is unreasonable to expect the recall of previous experience, for the purpose of establishing goal-directed intent, to occur spontaneously. Instead, it is likely influenced by input from the current context. Given the high prominence of the HC in representing both time and space, both egocentric and allocentric, it is likely that this information is provided by the HC.

Given the results of current and previous studies, reported from performance of the DNMP task, it is possible that the context sampling portion of SWM is accomplished through dynamic and on-going interactions between the PFC and the HC, orchestrated and enriched by the Re. The interactions allow for the comparison between representations of current and past experiences of the given environmental context, ultimately culminating in the PFC-driven development of an action plan. We can reason that trajectory representation is a function of spatial and temporal representations. Disrupting the vHC-mPFC pathway selectively disrupts the trajectory code, as the representation of current spatial context is fed forward from the HC to the mPFC.

The results of the current studies, however, extend these findings by demonstrating that in the absence of Re and dHC-Re neural activity, performance is disrupted almost entirely. These findings suggest that inhibiting communication from the HC to the PFC, particularly through the Re, cuts off vital representations of current
context necessary for making sense of the contextual relevance of previous experience, or possible even recalling it at all. Additionally, impairing performance by inhibiting mPFC-Re input suggests that the interactions between representations of previous and current experience is required for successful SWM performance.

5.3 Information Retention

Following the context sampling portion of SWM comes the information retention portion, constituted in the DNMP task by a delay period. The mechanisms supporting this phenomenon are challenging to clearly characterize. One possibility is that retention of contextual information across the delay period is achieved through sustained neuronal activity. It is also possible this is the point at which a behavioral plan is formed using the previously experienced contextual information. Alternatively, neuronal activity during the preceding context sampling phase could lead to a heightened state of excitability at synapses that then remain relatively silent, with the exception of spontaneous activity, in a form of short term synaptic plasticity\textsuperscript{139}. A third possibility is that information retention, strictly speaking, is not an independent phenomenon. Instead, information is simply stored and recalled, as driven by the other portions of the SWM timeline, such that no maintenance of the information is required.

In the current studies, there were no deficits associated with inhibiting Re, mPFC-Re, or dHC-Re activity during the delay period. However, there is evidence of sustained delay-period activity within the PFC\textsuperscript{140,141}. At first consideration, these results may seem to support the third possible explanation. If the PFC-Re-HC circuit is responsible for supporting SWM activities, with active information retention being one of those activities, then delay-period disruptions at least one of the pathways
ought to have impaired performance. However, this conclusion is premature, and even unlikely. There are specific pathways within this tri-regional circuit that were not examined in the current studies. It is possible that information retention is supported by those pathways, instead.

The first possibility, of sustained neuronal activity during the entirety of the delay, is also not likely. Such activity during the millisecond scale timeline in other working memory tasks would be too energy expensive to sustain for 20 continuous seconds. Furthermore, it seems unlikely that the tri-regional circuit communicates so heavily during the rest of the task, but does nothing task-relevant during a 20-second period of sustained activity. If that sustained activity were functionally relevant, and communicated across the circuit, silencing the Re would have impaired performance.

Reports of sustained delay period activity and the absence of circuit-wide contributions during the delay period must be reconciled. The most likely explanation is that information retention is supported by ‘activity-silent’ synapses in the prefrontal cortex. Stokes, 2015, argued that PFC neuronal populations code for contextual information that can be represented by a dynamic state, consisting of constant and fluid changes through multi-dimensional space. This activity alters the functional connectivity within the activated population, in a pattern-specific way, possibly by inducing short term plasticity mechanisms. Once that initial dynamic activity quiets, perhaps as the context sampling portion ends, the pattern-specific changes in functional connectivity remain, while the population goes relatively silent into a low-energy state. Spontaneous, rather than sustained, bursts of activity are subject to that specific pattern, demonstrating bursts of sequenced replay that may account for the
sustained activity reported in some working memory studies. As the delay period
draws to an end, the activity ramps in patterns representative of goal-direction.

Evidence for transitions through these dynamic and low-energy neuronal
activity states comes primarily from recordings of the PFC. In the current studies, no
performance deficits were associated with delay-period suppression of either the Re or
the dHC-Re pathway. Therefore, it is clear that the PFC is able to accomplish this
process without input from the dHC or the Re. Additionally, in an earlier study, no
disrupted trajectory or traversal PFC codes were evident as a function of delay period
vHC-mPFC suppression. These findings suggest that, with respect to this particular
circuit, the mPFC is likely able to accomplish information retention independently.

5.4 Pathway-specific Contributions to Memory-guided, Goal-directed
Navigation

By directly examining three distinct pathways during each portion of the SWM
timeline, the current studies demonstrated that there are notable differences in the way
the circuit supports SWM. Context sample was shown to require the dynamic
contributions of the entire circuit. By contrast, we showed that only the mPFC-Re
pathway was necessary for supporting the representation application portion. Finally,
suppressing the mPFC-Re pathway during choice traversal impaired performance.
Two important conclusions can be made from these findings. Firstly, the PFC-Re-HC
tri-regional circuit distinguishes between the context sampling and representation
application phases of SWM. Secondly, that while context sampling likely requires
dynamic interactions across the whole system, there is a very specific flow of
information required for the application of behaviorally-relevant SWM
representations.
In one study\textsuperscript{142}, researchers used the DNMP task to investigate the relationship between PFC and HC activity. Behaviorally, they noted that rats reliably ran slower during the choice traversal than the sample traversal. The representation application portion of the SWM timeline, as we have defined it, requires that representations of current context be compared both to the immediately preceding traversal and to earlier experiences with the task, so as to allow for an informed navigational plan to culminate in a rewarded correct choice. This first finding suggests that this process may require more time and attention than sampling the current environmental context alone. Next, theta-band power was equally strong during both the sample and choice traversals in the dHC\textsuperscript{142}. By contrast, vHC theta power during the sample traversal was significantly lower than during the choice traversal. They further reported that dHC-mPFC coherence was greater during the choice traversal than during the sample traversal. Theta power in both the dHC and vHC were both more highly correlated with mPFC theta power during the choice traversal than during the sample traversal. However, while vHC-mPFC coherence appeared to be independent of vHC-dHC coherence, dHC-mPFC coherence only appeared to be heightened when correlated with moments of strong vHC-mPFC coherence. When controlling for dHC activity, only vHC and mPFC theta power were more highly correlated during the choice phase, as compared to the sample phase. Finally, silencing the vHC with muscimol disrupted coherence and theta power correlations between the HC and mPFC. Additionally, only the ventral portion of the HC has direct projections to the mPFC. By contrast, while both the vHC and dHC connect through the Re, the dHC does not directly project to the mPFC\textsuperscript{143}. Taken with the results of the current study, in which no SWM performance impairment was associated with dHC-Re suppression, these
findings suggest that there is a unique directionality to the direct flow of information from the HC to the PFC that is distinct to the choice traversal; the representation application portion of SWM.

In further support of this notion, optogenetic suppression was also used during the DNMP task to investigate another input pathway in mice. Due to the contributions of the medial entorhinal cortex (MEC) to spatial cognition and influencing HC spatial representations, the MEC-dHC pathway should contribute to SWM. Firstly, power in the high gamma band (60-80Hz) was increased in the MEC during the choice traversal, relative to the sample traversal, but only at the decision-point of the maze. These high-gamma events were associated with correct choices. Furthermore, dHC high-gamma power and firing rate were reduced by optogenetically suppressing the MEC-dHC pathway, in anesthetized mice. In behaving animals, MEC-dHC suppression, only during the choice traversal, impaired performance and reduced the number of synchronized high gamma incidents at the T-junction. These findings further implicate a specific directionality to the flow of information during the choice traversal.

The choice traversal constitutes the representation application portion of our SWM timeline. Contextual information, such as current representations of time and space, must be constructed. Representations of the same information from the immediately preceding traversal must be recalled and compared to the current context. The aggregated representation is compared to previous experiences with learning the rules of the task and the maze, so that a navigational plan can be formulated. The decision must then be executed, and the animal can determine, based the presence or
absence of reward, whether their decision was correct before reconsolidating and/or their representations of the rules.

It is likely that the HC constructs representations of the current context during the earlier moments of this choice traversal, perhaps as early as the removal of the barricade after the delay period. That information can flow directly to the PFC, via the vHC, where representations of a plan of action and the retained information from the previous traversal are organized. As the animal approaches the decision-point, HC representations of events in time and space are likely refined by executive output from the mPFC, via the Re, and by an updated stream of processed sensory information from the EC. It is the looping of information between the PFC and HC with contributions from the Re and EC, and the aggregation of behaviorally relevant context into episodic representations in the HC\textsuperscript{131}, that is critical to SWM performance. Disrupting the flow of information at any point along that specifically demarcated pathway, such as the vHC-mPFC pathway, the mPFC-Re pathway, or the MEC-dHC pathway, is sufficient to impair performance accuracy.

5.5 Implications for Future Directions

Several critical components to the dynamics of this circuit must next be investigated. Firstly, structures of the brain are highly interconnected, and it is not necessarily safe to assume that the most apparently direct and efficient pathways are those used by the brain to accomplish its computations. Further investigations should expand on these findings by probing other pathways during this task. Additionally, before definitively concluding that there is actually a completed loop of information flow, future investigations should probe the Re-dHC pathway during the sample, delay, and choice segments of the DNMP. Based on the current findings, we would
expect that suppressing the Re-dHC pathway only during the sample and choice traversals, but not during the delay period, would yield performance deficits.

Another open question remains regarding the neuronal computations within the dHC involved in aggregating streams of information during each traversal, but particularly during the choice traversal. Recordings of how dHC activities are differentially influenced by input from both the Re and the MEC, for example, during each traversal might lend insight into how the HC processes this information. Furthermore, it will be very important to know what the HC then does with these representations, and how its outputs are read-out by downstream structures.

5.6 Conclusion

To conclude, we can reflect back on the open questions about how the PFC-Re-HC tri-regional circuit supports SWM that the current study proposed to answer. The current results suggest that, in fact, the sample and choice traversals do constitute two entirely unique process in the SWM timeline, as different contributions and different activities appear to be involved in each portion. We have argued that the construction of contextual representations occurs primarily during the context sampling portion, while the representation application portion is constituted in the DNMP task by the choice traversal. Context sampling would appear to be supported by dynamic interactions across the entirety of the circuit, while applying constructed representations to guide behavior appears to be dependent a directionally specific flow of information. The current discussion has contributed significantly to our understanding of SWM by helping to characterize the unique contributions of distinct pathways within the circuit.
REFERENCES


24. McKenna, J. T. & Vertes, R. P. Afferent projections to nucleus reuniens of the


35. Ferbinteanu, J. & Shapiro, M. L. Prospective and retrospective memory coding


82. Peyrache, A., Khamassi, M., Benchenane, K., Wiener, S. I. & Battaglia, F. P. Replay of rule-learning related neural patterns in the prefrontal cortex during


94. Warden, M. R. *et al.* A prefrontal cortex-brainstem neuronal projection that


106. Sampath, D., Sathyanesan, M. & Newton, S. S. Cognitive dysfunction in major


117. Bokor, H., Csáki, Á., Kocsis, K. & Kiss, J. Cellular architecture of the nucleus reuniens thalami and its putative aspartatergic/glutamatergic projection to the


134. Bourne, J. N. & Harris, K. M. Coordination of size and number of excitatory and inhibitory synapses results in a balanced structural plasticity along mature hippocampal CA1 dendrites during LTP. *Hippocampus* (2011). doi:10.1002/hipo.20768


143. Varela, C., Kumar, S., Yang, J. Y. & Wilson, M. A. Anatomical substrates for

Appendix A

PERMISSION STATEMENT

Title: Optogenetic suppression of the nucleus reuniens selectively impairs encoding during spatial working memory

Author: David J.-N. Malsson, Zachary M. Gemzik, Amy L. Griffin

Publication: Neurobiology of Learning and Memory

Publisher: Elsevier

Date: November 2018

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Appendix B

APPROVED IACUC PROTOCOL

University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Application to use animals in Research
(New and 3-Yr submission)

<table>
<thead>
<tr>
<th>Title of Protocol: Neural Correlates of Spatial Working Memory</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUP Number: 1177-2016-0 ← (4 digits only — if new, leave blank)</td>
</tr>
<tr>
<td>Principal Investigator: Amy L. Griffin, Ph.D.</td>
</tr>
<tr>
<td>Common Name (Strain/Breed if Appropriate): Long Evans Hooded Rats</td>
</tr>
<tr>
<td>Genus Species: Rattus Norvegicus</td>
</tr>
<tr>
<td>Date of Submission: Aug. 22, 2016</td>
</tr>
</tbody>
</table>

Official Use Only

IACUC Approval Signature: [Signature]

Date of Approval: 10/1/16
**Principal Investigator Assurance**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.</td>
</tr>
<tr>
<td>2.</td>
<td>I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).</td>
</tr>
<tr>
<td>3.</td>
<td>I understand that the Attending Veterinarians or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.</td>
</tr>
<tr>
<td>4.</td>
<td>I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.</td>
</tr>
<tr>
<td>5.</td>
<td>I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.</td>
</tr>
<tr>
<td>6.</td>
<td>I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.</td>
</tr>
<tr>
<td>7.</td>
<td>I assure that any modifications to the protocol will be submitted to the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.</td>
</tr>
<tr>
<td>8.</td>
<td>I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.</td>
</tr>
<tr>
<td>9.</td>
<td>I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.</td>
</tr>
<tr>
<td>10.</td>
<td>I assure that the experimental design has been developed with consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.</td>
</tr>
<tr>
<td>11.</td>
<td>I assure that the proposed research does not unnecessarily duplicate previous experiments. <em>(Teaching Protocols, Including Cooperative Extension Demonstrations, Exempt)</em></td>
</tr>
<tr>
<td>12.</td>
<td>I understand that by signing, I agree to these assurances.</td>
</tr>
</tbody>
</table>

Signature of Principal Investigator: [Signature]  
Date: 8/22/16

Rev 2/2016
# NAMES OF ALL PERSONS WORKING ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only those procedures that have been approved by the IACUC.

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Amy Griffin</td>
<td></td>
</tr>
<tr>
<td>2. Alicia Edsall</td>
<td></td>
</tr>
<tr>
<td>3. Andrew Garcia</td>
<td></td>
</tr>
<tr>
<td>4. David Maison</td>
<td></td>
</tr>
<tr>
<td>5. Zachary Gemzik</td>
<td></td>
</tr>
<tr>
<td>6. Margaret Donahue</td>
<td></td>
</tr>
<tr>
<td>7. Ronald Phillips</td>
<td></td>
</tr>
<tr>
<td>8. John Slout</td>
<td></td>
</tr>
<tr>
<td>9. Samuel Amer</td>
<td></td>
</tr>
<tr>
<td>10. Morgan Gaylord</td>
<td></td>
</tr>
</tbody>
</table>

If after hours participation is required by students on project involving *agricultural animals*, please describe how this is handled and the times and days that students may be on site.

Click here to enter text.
The Animal Use Protocol form has been developed to facilitate review of requests for specific research, teaching, or biological testing projects. The review process has been designed to communicate methods and materials for using animals through administrative officials and attending veterinarians to the Institutional Animal Care and Use Committee (IACUC). This process will help assure that provisions are made for compliance with the Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals.

Please read this form carefully and fill out all sections. Failure to do so may delay the review of this application. Sections that do not apply to your research must be marked “NA” for “Not Applicable.”

This application form must be used for all NEW or THREE-YEAR RENEWAL protocols.

*All answers are to be completed using Arial 12 size font.*

All questions must be answered in their respective boxes and NOT as attachments at the end of this form.

Please complete any relevant addenda:
- Hybridoma/Monoclonal Antibodies (“B”)
- Polyclonal Antibodies (“C”)
- **Survival Surgery (“D”)**
- Non-Survival Surgery (“E”)
- Wildlife Research (“F”)

If help is needed with these forms, contact the IACUC Coordinator at extension 2616, the Facility Manager at extension 2400 or the Attending Veterinarian at extension 2980.
1. Principal Investigator Information:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Name:</td>
<td>Amy Griffin</td>
</tr>
<tr>
<td>b. University/Company:</td>
<td>University of Delaware</td>
</tr>
<tr>
<td>c. Department:</td>
<td>Psychological and Brain Sciences</td>
</tr>
<tr>
<td>d. Building/Room:</td>
<td>Wolf 113</td>
</tr>
<tr>
<td>e. Office Phone:</td>
<td>X 2575</td>
</tr>
<tr>
<td>f. Lab Phone(s):</td>
<td>X 4695</td>
</tr>
<tr>
<td>g. Home Phone:</td>
<td>Click here to enter text</td>
</tr>
<tr>
<td>h. Mobile Phone:</td>
<td>617-833-3864</td>
</tr>
<tr>
<td>i. E-Mail Address:</td>
<td><a href="mailto:amygriff@psych.udel.edu">amygriff@psych.udel.edu</a></td>
</tr>
</tbody>
</table>

2. Protocol Status:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>a. ☐ New Protocol</td>
<td>☒ Re-submission due to three (3) completed years. If re-submission, enter Protocol Number: Click here to enter text.</td>
</tr>
<tr>
<td>b. ☒ Research</td>
<td>☐ Teaching or Cooperative Extension</td>
</tr>
<tr>
<td>c. ☒ Laboratory Animals</td>
<td>☐ Wildlife</td>
</tr>
<tr>
<td></td>
<td>If “Wildlife” please complete Addendum “f”</td>
</tr>
<tr>
<td></td>
<td>For agricultural animal protocols, please list the name and contact information for veterinarian who is on-call. A copy of the protocol should be shared with the veterinarian Click here to enter text.</td>
</tr>
<tr>
<td>d. Proposed Start Date:</td>
<td>November 10, 2016</td>
</tr>
<tr>
<td>e. Proposed Completion Date:</td>
<td>November 10, 2019</td>
</tr>
<tr>
<td>f. Funding Source:</td>
<td>National Institutes of Mental Health</td>
</tr>
<tr>
<td>g. Award Number if applicable:</td>
<td>R01MH102394</td>
</tr>
</tbody>
</table>

Rev 5/2/2016
3. Non-Scientific Summary: In language understandable to a high-school senior, very briefly describe the goals and significance of this study.

a. Specific Scientific Goals: The goal of the current project is to investigate the neural processes involved in learning and memory. Rat spatial memory tasks have been widely used to better understand how memories are formed in the brain. These tasks capitalize on the natural tendency for rats to forage for food and remember the location of existing food stores. By recording neural activity during spatial exploration, we are able to directly observe changes that occur in the brain while rats encode new memories. Additionally, we can design our tasks to also observe neural activity that occurs when the rat accesses an established memory and uses that knowledge to guide his behavior. Previous studies have shown that the hippocampus is crucial both for forming new memories and accessing old memories. The medial prefrontal cortex (mPFC) is also important in memory-guided behavior, but is also crucial for putting plans into action after a memory is retrieved. Recent work from our lab (Hallock, Weng and Griffin, 2016) shows that the ventral midline thalamic nucleus reuniens (Re) is an important orchestrator of hippocampal-prefrontal communication in the form of oscillatory synchrony, when oscillations coordinate neural activity across brain regions. However, there are still many unanswered questions about how the Re modulates activity within the hippocampal-prefrontal circuit. The specific goal of the current project is to investigate how the Re coordinates activity in the hippocampus and the mPFC during working memory by (1) recording activity from these three structures simultaneously and (2) by manipulating the hippocampus-Re-mPFC circuit using optogenetic techniques, which allows neural activity to be bidirectionally controlled by expressing light-sensitive channels in the region of interest. The temporal precision afforded by the latest generations of the microbial opsins toolkit, in conjunction with the anatomical specificity enabled by a projection specific suppression approach, allows for within-circuit manipulation at a time scale relevant to working-memory. The adeno-associated viral vectors (AAV5) described below will be targeted to cell bodies of the Re, subiculum, the output region of the hippocampus, and medial prefrontal cortex (depending on the experimental group) in order to manipulate neural activity on a millisecond timescale.

b. Significance of this Research or Teaching/Cooperative Extension Demonstration: (including the possible benefits to human and/or animal health, the advancement of scientific knowledge, or the betterment of society): Rat spatial working memory is a well-characterized and widely utilized animal model of memory. The hippocampus and related structures are remarkably conserved across evolution. Therefore, the current project will help us better understand how different memory systems in the brain interact to produce memory-guided behavior. Specifically, the information gained from the current project will help us better understand how memory systems compensate when certain structures are damaged.
or diseased. This approach will aid in developing new approaches to memory disorders which will lead to the design and implementation of treatments that strengthen existing structures when one or more parts of the memory system are compromised.

5. **Experimental Design**: Explain the experimental design. This description should allow the IACUC to understand fully the experimental course of an animal or group of animals from its entry into the experiment to the endpoint of the study.

The inclusion of flow charts, diagrams, and/or tables are greatly encouraged to explain experimental design or sequential events.

Be sure to include all animal events and related details, i.e.,

- **All Procedures**—bleedings, injections, surgical procedures, euthanasia, etc.
- **Procedural details**—number of animals involved in procedure, approximate animal weight, if relevant (for injections, bleeding, etc.), route, frequency, volume, etc.
- **Names of surgical procedures** (but reserve the surgical details for the proper Surgical Addenda)
- **Monitoring**—observations, measurements (animal weight, tumor size, etc)
- **Monitoring details**—criteria, frequency, names of personnel monitoring, conditions for removing an animal from the study, etc.
- **Endpoints**—include endpoints for the animals/study and how will they be determined.

(Describe):

**Subjects**

Long Evans hooded rats will be used for all procedures. Subjects will range in 3 to 9 months old at the beginning of the experiment. Both male and female rats will be used.

**Pretraining**

All animals will be brought into the laboratory for 4 - 6 hours per day for at least 5 days prior to beginning the experiment. The rats will remain in their home cages except for 20 to 30 minutes, during which time they will be handled by the experimenter. The rats will be handled every day until they show signs of being comfortable with the handling process (i.e. eating treats on the experimenter’s lap and the absence of defecation, urination and struggling). The rats will then be placed in the recording environment (small platform, open field, or maze) and allowed to forage for food for one hour per day for 5 days. In some cases, instead of foraging, the rats will be trained on a preliminary behavioral task.

**Control of neural activity with optogenetics**

Two AAV vectors will be obtained from the UNC Vector Core. An adeno-associated viral construct of titer exceeding $10^4$ 200 vg/ml (pAAV5-CAG-ArchT-GFP), containing the following components arranged sequentially downstream of the left-inverted terminal
repeat (ITR) of AAV5: cytomegalovirus (CMV) enhancer fused to the chicken beta-actin promoter (CAG), archaerhodopsin T1006 fused to green fluorescent protein (ArchT-GFP), woodchuck hepatitis posttranscriptional regulatory element (WPRE), SV4 PA terminator sequence and right-ITR, was used as the opsins-positive construct. The opsins-negative construct of titer exceeding 10⁴/12 vgl/ml (pAAV5-CAG-GFP) containing the following components arranged sequentially downstream of the left-inverted terminal repeat (ITR) of AAV5: cytomegalovirus (CMV) enhancer fused to the chicken beta-actin promoter (CAG), green fluorescent protein (GFP), woodchuck hepatitis posttranscriptional regulatory element (WPRE), SV4 PA terminator sequence and right-ITR. An AAV5 vector was chosen for this experiment to maximize cell transduction and subsequently axonal protein expression. High levels of axonal opsin expression are necessary to drive sufficient hyperpolarizing currents to silence terminal transmission, thus by delivering transgenes in a vector with high central nervous system (CNS) tissue tropism we were able to maximize the amount of RE synapsing neurons transduced (Paterna et al. 2004. Wu et al. 2006. Viruses (either control or pAAV5-CAG-ArchT-GFP) will be pressure-injected using a Harvard Apparatus PHD 2200 programmable syringe pump interfaced to a micropipette. The viral suspension will be injected at a flow rate of 0.1 μl/min for 5 min.

Surgery
Rats will then undergo survival surgery (see Addendum D), where they will be (1) implanted with a microdrive containing multiple adjustable electrodes; and/or (2) injected with an AAV in subiculum, mPFC, or Re and implanted with a fiberoptic “stub” attached to a ferrule that allows us to attach the patch cable for optical stimulation. In some rats, an optrode, a device used to deliver the optical stimulation and record LFPs from the same site (See Wang et al., 2012) will be implanted over the transduction site. After surgery, the rats will be allowed to recover in their home cages for 7–10 days, and will be checked and treated daily for signs of distress or infection. Two survival surgeries will be performed for animals that will be used for in vivo extracellular recordings in combination with optogenetic manipulations. These animals will undergo an initial survival surgery, during which the viral vector containing the optogenetic construct will be intracranially infused. The incision site will be stitched together, and the animal will recover post-surgically for a period of 4-5 weeks while the virus incubates. Following this 4-5 week period, the animal will undergo a second survival surgery, during which a microdrive array will be implanted for extracellular recordings. The 4-5 week incubation period required for complete viral transduction of neurons in a target brain area could cause problems if the microdrive was implanted when the virus was infused. We typically record from one rat for a period of 4-8 weeks. If the microdrive was implanted at the time of viral infusion, this means that the microdrive would be implanted for a period of 8-13 weeks (incubation time + recording time). This unusually long time period could lead to increased risk of infection, decreased microdrive stability, and poorer recording quality due to tetrode deterioration. Rats will be group-housed until they are put on food restriction. Singly-housed rats will be given enrichment toys in their cages.

Recording of behavior and neural data
Recording of behavior and/or neural activity will then begin. For each recording session, the rat will be placed in the recording environment, where the rat will perform one or more
of the tasks described below. For rats with implanted electrodes, the electrodes will be adjusted after the recording session so that the neural recording will have time to stabilize for the next recording session. For rats with optical fiber implants, the ferrule will be connected to a patch cable allowing for optical stimulation.

**Behavioral tasks**
Rats will perform one of 2 behavioral tasks.
1. Conditional discrimination - floor inserts on a T-maze signal whether the rat must turn left or right at the T junction in order to receive a food reward.
2. T-maze alternation – Rats must alternate from the left to the right goal area of a T-maze in order to receive food reward. In some cases, the trials are separated by a delay period.

The proposed experiments will use two different viruses to control the activity of Ret neurons or Re-projecting axon terminals from mPFC or subiculum: ChR2 transduction followed by blue-light stimulation to activate activity, and archeaodopsin (ArchT) transduction followed by green-light stimulation to suppress activity. Only one virus will be injected into each rat. For optical stimulation we have two different stimulation rigs. One rig is the Plexon Plexbright system that uses ultrabright LEDs to deliver light through a fiber optic cable. The other rig uses a laser diode light source (Doric) that interfaces with the Digital Lynx acquisition system and Cheetah software to control the laser with millisecond precision and insert event flags corresponding to laser onset and offset into the raw data file. All lab personnel have done laser safety training and rDNA training and all biosafety protocols are in place for the proposed work.

**Perfusion**
After the final recording session, the rat will be anesthetized with isoflurane, given an overdose of barbiturate anesthesia euthanasia solution (IP) and perfused intracardially with saline and formalin or 4% paraformaldehyde. The brain will then be removed for the histological verification of the recording or fiber/virus injection site.

**Immunohistochemistry**

The brain will be then removed and placed in a 4% paraformaldehyde solution. After soaking in paraformaldehyde for 1 to 2 days, the brains will be transferred to a sucrose solution. After sinking, indicating that the brain tissue was saturated, the brains will be frozen and sectioned (20-40 µm) using a cryostat or microtome. The sections were mounted on slides and prepared for immunohistochemistry.

For immunohistochemistry slides will go through three five minute 1x PBS (pH 7.2-7.4) washes. Slides will then be incubated at room temperature for one hour with a 5% goat serum/3% Triton X/PBS blocking buffer solution. After this incubation slides will be incubated for 24 hours in a cold room with a primary antibody solution (anti-GFP rabbit serum and blocking buffer in a 1/300 dilution). After 24 hours slides will be removed from the cold room and rinsed three times with 1x PBS (pH 7.2-7.4) for five minutes per rinse. The secondary antibody solution (goat anti-rabbit IgG and blocking buffer in a 1/300 dilution) will then be applied to the slides and allowed to incubate in the dark at room temperature for one hour. After the secondary incubation period slides will be rinsed three times with 1x PBS (pH
7.2-7.4) for five minutes per rinse, and were set out to air dry (about 15 minutes). Once dry, Prolong Gold antifade reagent will be applied to the sections and slides cover slipped. Slides will be set in the dark for 24 hours to cure before they are sealed. All antibodies will be obtained from Life Technologies. For all rinses and incubations, approximately 50-75uL of solution will be applied to each brain section, and slides placed on an orbital shaker to ensure all sections receive even exposure to the solutions. After staining, slides will be imaged using confocal or fluorescence microscopy to identify viral spread at infusion sites and axonal protein expression.

Flowchart of the experiment
Handling/ Pretraining (at least 10 days) – Surgery – Recovery (7-10 days) – Infusion/ Adjustment of electrodes and Recording during task performance (up to 90 days) – Perfusion and Histology

Number of animals per procedure (TOTAL = 210 animals)

<table>
<thead>
<tr>
<th>Procedure</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microdrive/fiber implant + virus injection</td>
<td>30</td>
</tr>
<tr>
<td>Fiber implant + virus injection</td>
<td>180</td>
</tr>
<tr>
<td><strong>ArchT</strong></td>
<td></td>
</tr>
<tr>
<td>opsin+</td>
<td>16</td>
</tr>
<tr>
<td>opsin-</td>
<td>14</td>
</tr>
<tr>
<td><strong>Chr2</strong></td>
<td></td>
</tr>
<tr>
<td>opsin+</td>
<td>16</td>
</tr>
<tr>
<td>opsin-</td>
<td>14</td>
</tr>
<tr>
<td><strong>RF</strong></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
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<tr>
<td>14</td>
<td></td>
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<tr>
<td><strong>mPFC</strong></td>
<td></td>
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<tr>
<td>16</td>
<td></td>
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<tr>
<td>14</td>
<td></td>
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<tr>
<td><strong>Sub</strong></td>
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<tr>
<td>16</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

For the fiber implant+ virus injection experiments, we are requesting an additional 4 animals per group in the experimental groups and 2 animals per group in the control groups in the event that animals need to be excluded from the study, for example due to incorrect virus or fiber placements.

For the Microdrive/fiber implant + virus injection experiments, there will be 20 opsin+ (10 rats for ArchT and 10 rats for Chr2) and 10 opsin- controls (5 rats for each virus).

6. Administration of compounds

<table>
<thead>
<tr>
<th>Drug name or class of drug</th>
<th>Volume</th>
<th>Dose or range of doses</th>
<th>Route (IP, IV, SC, IM, PO)</th>
<th>Frequency</th>
<th>Duration</th>
<th>Pharma-grade Yes or No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>Weight (Kg) x 0.05</td>
<td>0.05 mg/Kg</td>
<td>IM</td>
<td>Every hour</td>
<td>30-60 min.</td>
<td>Yes</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>N/A</td>
<td>1-3% in oxygen</td>
<td>Inhaled</td>
<td>Continuous</td>
<td>4-6 hours</td>
<td>Yes</td>
</tr>
<tr>
<td>------------</td>
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</tr>
<tr>
<td>Banamine</td>
<td>Weight (Kg) * 2.5 mg/Kg / 50 mg/ml</td>
<td>2.5 mg/Kg</td>
<td>IM</td>
<td>Once, end of surgery</td>
<td>12 hours</td>
<td>Yes</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>Weight (Kg) * 4 mg/Kg</td>
<td>4 mg/Kg</td>
<td>SC</td>
<td>Once, beginning of surgery</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>pAAV5-CAG-ArchT-GFP</td>
<td>.5 µL per site</td>
<td>$10^{12}$ vg/ml</td>
<td>Intracranial</td>
<td>1-6 injections</td>
<td>Each injection takes 5 minutes ($\mu$L per minute)</td>
<td>Yes/No</td>
</tr>
<tr>
<td>pAAV5-CAG-GFP</td>
<td>.5 µL per site</td>
<td>$10^{12}$ vg/ml</td>
<td>Intracranial</td>
<td>1-6 injections</td>
<td>Each injection takes 5 minutes ($\mu$L per minute)</td>
<td>Yes/No</td>
</tr>
</tbody>
</table>

If non-pharmaceutical grade compounds are used, they must be justified (such as pharmaceutical grade not available) and the method to ensure appropriate preparation must be described: (for example: pharmaceutical grade drugs are not available. Sterile saline will be used as a vehicle and the solution will be sterile-filtered. Pharmaceutical grade is not available. Phosphate-buffered saline will be used as a vehicle and the solution will be sterile-filtered.

7. Does this work involve surgery or antibody production ☐ Yes ☑ No

If yes, please complete Addendum B for hybridoma/murine antibody production, Addendum C for polyclonal antibody production, Addendum D for survival surgery and Addendum E for terminal surgery.
### REFINEMENT, REDUCTION & REPLACEMENT

When using animals for research, it is important to consider the three Rs: reduction, refinement, and replacement to reduce both animal distress and the number of animals used in the laboratory.

- **Reduction:** Minimizing the number of animals used
- **Refinement:** Using techniques and procedures to reduce pain and distress
- **Replacement:** Using non-animal methods or lower phylogenetic organisms

---

### 8. Justification for the Use of Animals (instead of *in vitro* methods)

*Check all that apply and explain:*

a. ☐ The complexity of the processes being studied cannot be duplicated or modeled in simpler systems: *(Explain):* Because we are investigating neural activity associated with learning and memory, the animals must be awake and performing a memory task while we collect the neural data. While computer models are useful to generate predictions about experimental work, awake behaving animals are needed to investigate the true processes that are occurring in the brain as a real live rat learns a task. Cell cultures are not appropriate because we are correlating behavior with neural activity and this is impossible to study in individual cells.

b. ☐ There is not enough information known about the processes being studied to design non-living models: *(Explain):* Click here to enter text.

c. ☐ Other: *(Explain):* Click here to enter text.

---

### 9. Justification for Species Appropriateness:

*Check all that apply and explain:*

a. ☐ A large database exists, allowing comparisons with previous data: *(Explain):* Click here to enter text.

b. ☐ The anatomy or physiology is uniquely suited to the study proposed: *(Explain):* Click here to enter text.

c. ☐ This is the lowest species on the phylogenetic scale suitable to the proposed study: *(Explain):* Click here to enter text.

---

Rev1 202/2016

#1177-2016-0
d. Other: *(Explain)*: Because there is a long history in our field of using rats in spatial working memory tasks, the paradigm is well-characterized and minimizes confounding variables that may compromise the data.

10. Justification for Number of Animals Requested: *(Note: numbers should include animals used for breeding and all animals born)*

a. □ Pilot study or preliminary project where group variances are unknown at the present time. Describe the information used to estimate how many animals will be needed. *(Only a limited number of animals will be permitted.)* *(Explain):* Click here to enter text.

b. □ Group sizes are determined statistically. Describe the statistical analysis used to estimate the number *(N)* of animals needed: N may be estimated from a power analysis for the most important measurement in the study, usually based on the expected size of the treatment effect, the standard error associated with the measurement, and the desired statistical power *(e.g., \( P < 0.05 \)). Data analysis methods should not be submitted unless directly applicable to the estimate of N.

   An online calculator may be found at: [http://www.math.uiowa.edu/~rlenth/Power/](http://www.math.uiowa.edu/~rlenth/Power/) or a stand-alone calculator that can be downloaded from [http://wwwpsycho.uni-duesseldorf.de/abteilungen/aap/gpower3](http://wwwpsycho.uni-duesseldorf.de/abteilungen/aap/gpower3).

 *(Explain):* We will need 12 animals per group. This is based on a power analysis from the online calculator noted above. The parameters were \( \sigma = 0.41 \), power = 0.8145, Difference of means = 0.5. The parameters for the online calculator were obtained from previous studies from our lab.

c. □ Group sizes are based on the quantity of harvested cells or the amount of tissue required for *in vitro* studies. Explain how much tissue is needed based on the number of experiments to be conducted and the amount of tissue you expect to obtain from each animal *(e.g., 10g of tissues are needed: Each animal can provide 2g. 10g x 2g per animal = 5 animals needed.)* *(Explain):* Click here to enter text.

d. □ Teaching or cooperative extension demonstration protocol. Specify the number of students in the class, the student to animal ratio and how that ratio was determined: Animal numbers should be minimized to the fullest extent possible without compromising the quality of the hands-on teaching experience for students or the health and welfare of the animals. *(Explain):* Click here to enter text.

e. □ Study involving feral or wild animals. Animals will be captured and released in an attempt to maximize the sample size within logistical constraints. Describe the process by which you estimate these numbers and estimate the precision needed. *(Explain):* Click here to enter text.
f. ☐ Observational, non-manipulative study. Animals will not be captured, their behavior will not be interfered with, and exact animal numbers cannot be predicted. *(Explain)*: Click here to enter text.

g. ☐ Product testing. The number of animals needed is based on FDA or USDA guidelines. Provide the citation from the regulations, the IND tracking number, or relevant FDA or USDA correspondence. *(Explain)*: Click here to enter text.

h. ☐ Other. Elaborate, indicating the method used to determine the group size. *(Explain)*: For neural recording experiments, we used journal articles utilizing similar methods as a guide for determining how many animals were appropriate to use for each manipulation. Power analysis is not appropriate for these studies because the results are reported as descriptions of categories of neural correlates instead of statistical differences.

### 11. Animals Requested:

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Genus and Species</th>
<th>Total Number of Animals for Three Years</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Long Evans hooded rats</td>
<td>Rattus norvegicus</td>
<td>210</td>
</tr>
<tr>
<td>2. Click here to enter text.</td>
<td></td>
<td></td>
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<tr>
<td>3. Click here to enter text.</td>
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<td></td>
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<tr>
<td>4. Click here to enter text.</td>
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<td></td>
</tr>
<tr>
<td>5. Click here to enter text.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 12. Where will animals be obtained and are there any special shipping requirements? Harlan or Charles River labs. No special shipping requirements.

If these are privately owned animals please attach an owner consent form

Are agricultural animals obtained from a non-traditional source such as poultry from a commercial production company or swine from commercial herd? ☐ Yes ☐ No
If yes, please describe how the animals are tested and determined to be free of diseases which could potentially infect other animals on site, and any special precautions, such as quarantine isolation housing that is required. Click here to enter text.

12. Where will animals be housed (or captured for wildlife)? OLAM

14. Will any untreated or non-manipulated animals be humanely euthanized, to obtain tissue, cells, etc.? □ Yes □ No

If Yes, list types of tissue, etc: Click here to enter text.

15. Dietary Manipulations □ Yes □ No

If Yes, list and explain (Note: If food or fluid will be restricted, describe method for assessing the health and well-being of the animals. Body weights must be recorded at least weekly. Amount earned (if animals work for food or fluid) during testing and amount freely given must be recorded. A scientific justification must be provided for departures from the recommendations of the Guide.) Rats will be fed once per day by our laboratory personnel. The rats will be kept at 90% of their free-feeding weight. The purpose of the food restriction is to ensure that the rats are sufficiently motivated to perform food-rewarded tasks. During the recording sessions, the rats will receive food rewards (sweet cereal or chocolate sprinkles). Rats will be weighed once per week.

16. Environmental Stress (e.g. cold, prolonged restraint, forced exercise, shock) □ Yes □ No

If Yes, list and explain: Click here to enter text.

17. Special Study Requirements or Exceptions to Standards: Please describe any special study requirements such as single housing of the animals, exemption from environmental enrichment, or special caging. After implantation of microdrives, there will need to be a water bottle attached to the side of the cage so that the rat can easily access water. We have already been using these water bottles in our previous protocol. Animals on food restriction need to be housed singly to ensure that each rat has access to its full ration of food.

17. Will any animal undergo anesthesia for any reason other than surgery? □ Yes □ No

If Yes,

a. List Procedures and Reason(s) for using anesthesia: In some cases, there may be need to repair the Microdrive or fiber stub implant. If so, the rat will be briefly anesthetized with isoflurane using that same procedures for anesthesia induction that are described in detail in Addendum D.
b. Check the type of anesthesia to be used.
   ☒ Isoflurane

   ☐ Injectable (For injectable, complete the following):

   Drug: Click here to enter text.
   Dose: Click here to enter text.
   Route: Click here to enter text.

HAZARDOUS AGENTS

18. Administration of Hazardous Chemicals, Drugs, Toxins, or Nanoparticles
   ☐ Yes  CAS#_____  ☒ No

   If Yes, describe hazards posed to personnel: Click here to enter text.

   Methods to control exposure: Click here to enter text.

   Methods of Disposal of Animals and Bedding: Click here to enter text.

19. Administration of radioactive materials  ☐ Yes  ☒ No

   a. Type to be used. Include radionuclide(s) and chemical form(s): Click here to enter text.

   b. Describe the practices and procedures to be followed for minimization of radiation exposure to
      workers and for the handling and disposal of contaminated materials associated with this study:
      (Include the methods for management of radioactive wastes and monitoring facility for radioactive
      contamination, if applicable.) Click here to enter text.

   c. Who will be responsible for the daily care of animals containing radioactive materials?
      Click here to enter text.

   d. Approval received from UD- Environmental Health and Safety? ☐ Yes  ☐ No  ☐ Pending
      Click here to enter text.

      Please attach a copy of any approvals or provide the approval number.
20. **Study of Irradiation in vivo**

- **Yes** (gamma irradiator? ☐ or x-ray irradiator? ☐)
- **No**

  a. Make, model, and location of irradiator to be used:

  Click here to enter text.

  b. Approval received from UD- Environmental Health and Safety? **Yes ☐ No ☐ Pending**

  Please attach a copy of any approvals or provide the approval number. Click here to enter text.

21. **Administration of Biological Agents** (eg microorganisms, recombinant DNA, **HUMAN** serum, tissue, cell lines, etc.) **Yes ☒ No ☐**

  Animal Biosafety Level ☒ 1 ☐ 2 ☐ 3 ☐ 4

  Source of Biological Agents: AAV5 from UNC vector core

| Describe hazards posed to personnel: | None |
| Methods to control exposure: | **Personal protective equipment** (gloves, lab coat, safety glasses) |
| Methods of Disposal of Animals and Bedding: | Animal carcasses will be incinerated. Bedding will be disposed of in chemical waste containers. |
| Approval received from UD- Institutional Biosafety Committee, and if required, the UD-Select Agent Committee? | **Yes ☒ No ☐ Pending** |

Please attach a copy of any approvals or provide the approval number. Annual renewal of rDNA approval is pending.

22. **Will tumor cells, tissue, sera, viral vectors or other biologies of RODENT origin – other than those isolated from rodents already housed in the facility – be administered to animals?**

- **Yes ☐ No ☒**

If Yes, this material must be tested for rodent pathogens and test results must be attached. (Please contact the **Attending Veterinarian** for details.)
23. Use of Genetically Engineered (GEM, transgenic, knockout) Animals

☐ Yes  ☒ No

If Yes, please describe any anticipated phenotypes that may cause pain or distress and any special care or monitoring that the animals will require.

Click here to enter text.

Does the proposed work involve creating new genetically modified animals, or involve crossing two genetically modified animals to produce offspring with a new genotype.

☐ Yes  ☒ No

Approval received from UD- Institutional Biosafety Committee?

☐ Yes  ☐ No  ☐ Pending  ☐ Exempt (breeding of two lines of genetically-modified rodents is exempt if 1) both parents can be housed under BL1 containment and 2) neither parent strain incorporates more than one half of the genome of an exogenous eukaryotic virus or incorporates a transgene under the control of a gammaretroviral long terminal repeat and 3) the rodent that results from the breeding is not expected to contain more than one half of an exogenous viral genome)

Please attach a copy of any approvals or provide the approval number.

______________________ Click here to enter text ______________________

24. Pain Category: (please mark one)

<table>
<thead>
<tr>
<th>USDA PAIN CATEGORY: (Note change of categories from previous form)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Category</strong></td>
</tr>
<tr>
<td>☐ B</td>
</tr>
<tr>
<td>☐ C</td>
</tr>
<tr>
<td>☒ D</td>
</tr>
<tr>
<td>☐ E</td>
</tr>
</tbody>
</table>

25. If animals may experience pain or distress, (for example, animal challenge studies using a pathogenic disease agent) please include how they will be monitored, frequency of observation, and potential treatments (note: for survival surgery procedures this will be described in addendum D and does not need to be repeated here) See Addendum D

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26. Please describe criteria for when an animal will be euthanized (humane endpoints – possible examples include 20% weight loss, ulceration of subcutaneous tumors, difficulty ambulating, hunched posture):

Loss of microdrive or fiber stub implant. Any signs of significant distress (significant weight loss, 20% or more), difficulty ambulating, hunched posture, reduced activity that does not improve with appropriate treatment. The UD veterinarian will be consulted for advice on what treatment options are available.

Alternatives to Pain and Distress

27. If you have indicated that animals in your study experience pain or distress (category D or E), even if it will be fully alleviated, please mark the appropriate check boxes below and fill in the requested information for each item marked. (Note: If the pain category is B or C, please skip to question 28)

You must conduct at least two (2) searches.

I have considered alternatives to the use of animals in my study. Alternatives refer to methods or approaches which result in refinement of procedures which lessen pain and/or distress; reduction in numbers of animals required; or replacement of animals with non-whole-animal systems or replacement of one animal species with another, particularly if the substituted species is non-mammalian or invertebrate. I have used the following methods and sources to search for alternatives:

Note: You may need to do more than one search per database to look for alternatives if there are multiple procedures that may cause pain and/or distress.

Database Used:

☐ Medline
☐ Toxline
☐ Biois
☐ Agricola
☐ CAB Abstracts
☐ Other (Specify): Click here to enter text.

Date of Search: Aug 22, 2016

Years Covered: 1959 to present

Keywords Used (must include the word alternative): alternatives, neurophysiology, memory, stereotaxic injection

Number of Papers Found: 33

Discussion of the Relevancy of the Papers Found: In both of these papers, “alternatives” refers to alternative methodological changes to the stereotaxic surgery procedure rather than alternatives to performing stereotaxic surgery. Interpretation of theoretical approaches rather than alternatives to using animals.
Database Used:
- Medline
- Toxicology
- Bioisys
- Other (Specify): 

Date of Search: Aug. 22, 2016

Years Covered: 1962 to present

Keywords Used (must include the word alternative): alternatives neurophysiology memoeys stereotoxic injection

Number of Papers Found: 134

Discussion of the Relevancy of the Papers Found: In none of the papers found was the word "alternative" used in the context of alternatives to using rats in electrophysiological experiments. In most cases, the word "alternative" referred to an alternative methodology or alternative interpretation of a data set. This paper used stereotoxic injection. The word "alternative" was used in the context of alternative explanation of Alzheimer's disease pathology.

Unnecessary Duplication of Work.

28. Activities involving animals must not unnecessarily duplicate previous experiments performed by you or others. Provide a written narrative that assures that the activities of this project comply with this requirement and support this assurance by performing a literature search.

The search should return, at minimum, the related previous work from your laboratory.

You must conduct at least two (2) searches.

(NOT REQUIRED FOR TEACHING PROTOCOLS)

Note: You may need to do more than one search per database to look for duplication of work, especially if you are doing more than one experiment.

Database Used:
- Medline
- Toxicology
- Bioisys
- Other (Specify): 

Date of Search: Aug. 22, 2016

Years Covered: 1962 to present

Keywords Used: optogenetics, reuniens
Number of Papers Found: 2

Discussion of the Relevancy of the Papers Found: These two papers are very relevant to our work in that they use optogenetics to manipulate reunions activity. However neither of these papers uses a projection-targeting approach to compare the effects of manipulating two different inputs to reunions.

Database Used:
- ☒ Medline
- ☐ Agricola
- ☒ Toxline
- ☐ CAB Abstracts
- ☐ Biosis
- ☐ Other (Specify): Click here to enter text.

Date of Search: Aug. 22, 2016

Years Covered: 1965 to present

Keywords Used: optogenetics, reunions

Number of Papers Found: 3

Discussion of the Relevancy of the Papers Found: All 3 of these citations were from the NIH database, describing work that is proposed in a grant. In all 3 cases, the work was related to the work that we will do, but does not significantly overlap. Two of the citations propose to investigate fear memory generalization and one will compare input from the Re and the entorhinal cortex on hippocampal activity in mice without recording behavior.

Disposition of Animals
29. What is the expected disposition of animals at the end of the experiments?
   (Check all that apply):
   ☐ Euthanized - If an infectious disease studies - carcasses decontaminated by ☐ incineration
   ☐ composting ☐ other Click here to enter text.

☐ Maintained
☐ Released (Wildlife Only)
☐ Other (Specify): Click here to enter text.

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#1177-2016-0
### 30. Euthanasia

Select methods that will be used in case of emergency and/or at the end of the procedure/experiment.

**NOTE:**
- Methods must be approved by the AVMA or must be scientifically justified.
- A “Primary” and “Secondary” method must be selected (UD Double Kill Policy).
- **If different methods will be used for different groups** of animals, indicate the group after the procedure (e.g., write “Neonates” after Decapitation, “Adults” after CO₂, “Terminal Surgery Animals” after Isoflurane Anesthesia Overdose, etc.).

- Animals will NOT be under anesthesia when euthanasia is performed.
- Animals will be under anesthesia when euthanasia is performed. *(Check drug used below):*

#### Isoflurane

- Injectable *(Complete the following):*
  - **Drug:** [Click here to enter text.]
  - **Dose:** [Click here to enter text.]
  - **Route:** [Click here to enter text.]

### PRIMARY method(s) of euthanasia

- **CO₂** by compressed gas cylinder *(Not for animals already under anesthesia or neonates)*

- **Barbiturate Euthanasia Solution - Injectable ≥150mg/kg** *(Check route below):*
  - **IV**
  - **IP**
  - **IC**

- Isoflurane Anesthesia Overdose - Inhalant

- **Cervical Dislocation** *(acceptable with anesthesia, or for poultry, without anesthesia if personnel are trained)*

- Decapitation *(only under anesthesia or neonates)*

- Exsanguination or Perfusion *(only under anesthesia)*

- Incision of Chest Cavity – Bilateral Pneumothorax *(only under anesthesia)*

- Pithing *(only under anesthesia) (amphibians, reptiles only)*

- Removal of Vital Organ(s) *(only under anesthesia) (Check all that apply):*
  - **Brain**
  - **Kidneys**

Rev22/02/2016
- Heart
- GI Tract
- Liver
- Lungs
- Other Vital Organ(s) – *(Specify)*: Click here to enter text.

- Other Method of Euthanasia: *(Describe and Scientifically Justify):*  

**SECONDARY method(s) of euthanasia that will be used to ensure that the animal does not survive:**

- Cervical Dislocation
- Decapitation
- Exsanguination or Perfusion
- Incision of Chest Cavity – Bilateral Pneumothorax
- Barbiturate Euthanasia Solution - Injectable ≥150mg/kg *(Check route below):*
  - IV
  - IP
  - IC
- Pithing – Double pithing required *(fish, amphibians, reptiles only)*
- Monitor for lack of respiration and heart beat *(Agricultural animals only)*

**Removal of Vital Organ(s):** *(Check all that apply):*

- Brain
- Kidneys
- Heart
- GI Tract
- Liver
- Lungs
- Other Vital Organ(s) – *(Specify):* Click here to enter text.

- Other Method of Euthanasia: *(Describe and Scientifically Justify):* Click here to enter text.

**Personnel and Training**

**31. Personnel involved in Protocol *(Include Principal Investigator):*  

*Status: Indicate Prof, Post-Doc, Grad Student, Lab Manager, Research Assistant, Technician, etc.*
**Qualifications:** Include procedures this person is proficient in performing on proposed species and the time they have been doing the procedure. **Be specific** (e.g. sub-mandibular bleeding on mice-2yrs, performing castrations on mice and rats-1yr, tail-vein injections on mice-2yrs, etc.) **(If no experience, list who will train.)**

**Responsibilities:** Include all responsibilities this person will have with live animals on this protocol, including euthanizing animals.

<table>
<thead>
<tr>
<th>Name</th>
<th>E-mail</th>
<th>Office phone number</th>
<th>Home/Cell phone number</th>
<th>Received IACUC-required training</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amy Griffin</td>
<td><a href="mailto:amygrff@psych.udel.edu">amygrff@psych.udel.edu</a></td>
<td>2575</td>
<td>617-833-3864</td>
<td>Yes ☒ No □</td>
</tr>
</tbody>
</table>

**Status:** Principal Investigator

Qualifications: Stereotaxic surgery, Training on behavioral tasks, Virus microinfusions, Electrophysiological recording, Optogenetic silencing, Perfusions, Euthanasia on rats – 13 years

Responsibilities: Conduct all lab procedures and train other laboratory personnel on lab techniques. Study planning and oversight.

<table>
<thead>
<tr>
<th>Name</th>
<th>E-mail</th>
<th>Office phone number</th>
<th>Home/Cell phone number</th>
<th>Received IACUC-required training</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrew Garcia</td>
<td><a href="mailto:awgarcia1@gmail.com">awgarcia1@gmail.com</a></td>
<td>4895</td>
<td>213-304-2198</td>
<td>Yes ☒ No □</td>
</tr>
</tbody>
</table>

**Status:** Graduate Student

Qualifications: Stereotaxic surgery, Training on behavioral tasks, Intracranial virus microinfusions, Electrophysiological recording, Perfusions, Euthanasia on rats – 2 years. Trained on all procedures by Amy Griffin and Henry Hallock (former grad student/postdoc).

Responsibilities: Conduct all lab procedures and train other laboratory personnel on lab techniques.

<table>
<thead>
<tr>
<th>Name</th>
<th>E-mail</th>
<th>Office phone number</th>
<th>Home/Cell phone number</th>
<th>Received IACUC-required training</th>
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</thead>
<tbody>
<tr>
<td>Alicia Edsall</td>
<td><a href="mailto:aedsall@psych.udel.edu">aedsall@psych.udel.edu</a></td>
<td>4685</td>
<td>484-464-7158</td>
<td>Yes ☒ No □</td>
</tr>
<tr>
<td>Name</td>
<td>E-mail</td>
<td>Office phone number</td>
<td>Home/Cell phone number</td>
<td>Received IACUC required training</td>
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<tr>
<td>-----------------</td>
<td>---------------------</td>
<td>---------------------</td>
<td>------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>David Maisson</td>
<td><a href="mailto:dmaisson@psych.udel.edu">dmaisson@psych.udel.edu</a></td>
<td>4895</td>
<td>310-867-1577</td>
<td>Yes ☑ No ☐</td>
</tr>
<tr>
<td>Margaret Donahue</td>
<td><a href="mailto:mdonahue@udel.edu">mdonahue@udel.edu</a></td>
<td>4895</td>
<td>401-297-7347</td>
<td>Yes ☑ No ☐</td>
</tr>
<tr>
<td>Ronald Phillips</td>
<td><a href="mailto:ronphil@udel.edu">ronphil@udel.edu</a></td>
<td>4895</td>
<td>(484) 919-9143</td>
<td>Yes ☑ No ☐</td>
</tr>
</tbody>
</table>
### Status: Undergraduate research assistant

**Qualifications:** Proficient at rat training, perfusions, histology and intracranial virus microinfusions. He is in the process of being trained on survival surgeries by David Maison.

**Responsibilities:** Rat handling, electrophysiology, training, microinfusions, surgeries (with supervision), perfusions and histology.

<table>
<thead>
<tr>
<th>Name</th>
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<th>Office phone number</th>
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<th>Received IACUC required training</th>
</tr>
</thead>
<tbody>
<tr>
<td>John Stout</td>
<td><a href="mailto:jhnyboy@udel.edu">jhnyboy@udel.edu</a></td>
<td>4895</td>
<td>914-262-3520</td>
<td>Yes ☑ No □</td>
</tr>
</tbody>
</table>

### Status: Undergraduate research assistant

**Qualifications:** Proficient at rat training, perfusions, histology and intracranial virus microinfusions. He is in the process of being trained on survival surgeries by David Maison.

**Responsibilities:** Rat handling, electrophysiology, training, microinfusions, surgeries (with supervision), perfusions and histology.

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<th>Received IACUC required training</th>
</tr>
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<tr>
<td>Samuel Amer</td>
<td><a href="mailto:seamer@udel.edu">seamer@udel.edu</a></td>
<td>4895</td>
<td>Click here to enter text</td>
<td>Yes ☑ No □</td>
</tr>
</tbody>
</table>

### Status: Research assistant

**Qualifications:** Proficient at rat training, perfusions, histology and intracranial virus microinfusions. He is in the process of being trained on survival surgeries by David Maison.

**Responsibilities:** Rat handling, electrophysiology, training, microinfusions, surgeries (with supervision), perfusions and histology.

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<th>Office phone number</th>
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<th>Received IACUC required training</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zachary Gemzik</td>
<td><a href="mailto:zgemzik@udel.edu">zgemzik@udel.edu</a></td>
<td>4895</td>
<td>610-306-1939</td>
<td>Yes ☑ No □</td>
</tr>
</tbody>
</table>

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103
Status: Research assistant

Qualifications: Proficient at rat training, perfusions, histology and intracranial virus microinfusions. He is in the process of being trained on survival surgeries by David Maisson.

Responsibilities: Rat handling, electrophysiology, training, microinfusions, surgeries (with supervision), perfusions and histology.
University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Research and Teaching

ADDENDUM “D”

Survival Surgery

(Please use a separate form for each surgical procedure and each species.)

<table>
<thead>
<tr>
<th>AUP Number: 1177-2016-0</th>
<th>(4 digits only — if new, leave blank)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project: Neural correlates of spatial working memory</td>
<td></td>
</tr>
</tbody>
</table>

General Information

1. Name of survival surgical procedure: Microdrive/Fiber optic stub implant with AAV injection

2. Reason for performing this procedure: To manipulate and record neural activity in freely moving rats

3. Species: Long Evans hooded rats

4. Total maximum number of animal undergoing this surgical procedure over 3 years: 210

5. Location of the surgery:
   a. Building: McKinly Lab, OLAM
   b. Room number: OLAM

6. Type of Surgery: (choose one)
   - [ ] Minor Operative Surgery
   - [X] Major Operative Surgery
     (Opening a body cavity, opening the cranium, or producing substantial impairment)
7. Will any animals undergo more than one MINOR survival surgery?

☐ Yes  ☐ No

*If Yes, complete the following:*

<table>
<thead>
<tr>
<th>Maximum number of surgeries an animal will undergo:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type(s) of surgeries that the animal will undergo:</td>
</tr>
<tr>
<td>Time interval between surgeries:</td>
</tr>
</tbody>
</table>

Justify need for multiple surgeries:

8. Will any animals undergo more than one MAJOR survival surgery?

(Strongly Discouraged)

☐ Yes  ☐ No

*If Yes, complete the following:*

<table>
<thead>
<tr>
<th>Maximum number of surgeries an animal will undergo:</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type(s) of surgeries that the animal will undergo:</td>
<td>Virus injection and Microdrive implant</td>
</tr>
<tr>
<td>Time interval between surgeries:</td>
<td>4-6 weeks</td>
</tr>
</tbody>
</table>

Justify need for multiple surgeries: The 4-5 week incubation period required for complete viral transduction of neurons in a target brain area could cause problems if the microdrive were implanted when the virus was infused. We typically record from one rat for a period of 4-6 weeks. If the microdrive were implanted at the time of viral infusion, this means that the microdrive would be implanted for a period of 8-13 weeks (incubation time + recording time). This unusually long time period could lead to increased risk of infection, decreased microdrive stability, and poorer recording quality due to tetrode deterioration.

**Medication and Fluid Administration**

*not anesthetics and analgesics*

9. Will neuromuscular blocking agent(s) be used?

☐ Yes  ☐ No

*If Yes, complete the following*
10. Will any drugs or agents (OTHER THAN anesthetics or analgesics) be administered during surgery (e.g. antibiotics, atropine, saline, specific drugs or agents as part of the experiment)?

Yes ☐ No ☐

If Yes, complete the following for each drug:

<table>
<thead>
<tr>
<th>Drug</th>
<th>atropine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>.05</td>
</tr>
<tr>
<td>Route</td>
<td>IM</td>
</tr>
<tr>
<td>When first administered</td>
<td>20 minutes before isoflurane induction</td>
</tr>
<tr>
<td>Frequency</td>
<td>Once</td>
</tr>
<tr>
<td>Purpose</td>
<td>Clear airway</td>
</tr>
</tbody>
</table>
### Pre-Surgical Procedures and Preparation

11. Sterilization of Instruments (check all that apply)

<table>
<thead>
<tr>
<th>Option</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>☐ Autoclave</td>
<td>Click here to enter text.</td>
</tr>
<tr>
<td>☐ Chemical Sterilization (specify agent)</td>
<td>Click here to enter text.</td>
</tr>
<tr>
<td>☒ Bead sterilization</td>
<td>Click here to enter text.</td>
</tr>
<tr>
<td>☐ Other (specify):</td>
<td>Click here to enter text.</td>
</tr>
</tbody>
</table>

12. Surgeon Preparation for Aseptic Technique (check all that apply)

<table>
<thead>
<tr>
<th>Option</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>☐ Surgical hand wash</td>
<td>☐ Sterile surgical gown</td>
</tr>
<tr>
<td>☒ Sterile surgical gloves</td>
<td>☐ Surgical Face Mask</td>
</tr>
<tr>
<td>☒ Clean Lab Coat (rats and mice only)</td>
<td>☒ Surgical Cap/bootsies</td>
</tr>
<tr>
<td>☐ Non-sterile exam gloves (rats and mice — minor procedures only)</td>
<td>☐ Other (list): Click here to enter text.</td>
</tr>
</tbody>
</table>

13. Will food be withheld prior to surgery? (not usually necessary for mice, rats, rabbits)

<table>
<thead>
<tr>
<th>Option</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>☒ No</td>
<td>☐ Yes</td>
</tr>
</tbody>
</table>

If Yes,

- Duration: Click here to enter text.
- Justification: Click here to enter text.

14. Will water be withheld prior to the surgery? (not usually necessary for mice, rats, rabbits)

<table>
<thead>
<tr>
<th>Option</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>☒ No</td>
<td>☐ Yes</td>
</tr>
</tbody>
</table>

If Yes,

- Duration: Click here to enter text.
- Justification: Click here to enter text.
**Anesthesia**

15. **Indicate type of anesthesia that will be used**: (complete the requested information)

- Isoflurane

  | % Induction: | 3 |
  | % Maintenance: | 0.5-2.5 |

- Injectable

  | Drug(s): | Click here to enter text. |
  | Dose (mg/kg): | Click here to enter text. |
  | Route: | Click here to enter text. |
  | Expected Duration of Agent: | Click here to enter text. |
  | Supplemental Dosing information (if needed) | Drug: Click here to enter text. |
  | | Dose: Click here to enter text. |
  | | Route: Click here to enter text. |

16. **Monitoring of Depth of Anesthesia** (check all that apply)

- ☒ Toe Pinch
- ☒ Tail Pinch
- ☐ Corneal Reflex
- ☒ Heart Rate
- ☐ Muscle Relaxation
- ☒ Respiration Rate
- ☐ EKG
- ☐ EEG
- ☐ Mucous membrane color and/or capillary refill time
- ☐ Other (specify): Click here to enter text.

**Surgical Procedure**

*Aseptic Technique must be used on ALL Animals*

17. **Animal Preparation**: (check all that apply)
<table>
<thead>
<tr>
<th>Hair Shaved</th>
<th>Surgical Scrub</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye Lubricant</td>
<td>Sterile drape</td>
</tr>
</tbody>
</table>

☐ Other (specify): Click here to enter text.

18. Procedure to Maintain Normal Body Temperature: (check all that apply)

☐ Warm Waterbed
☐ Heat pack/pad
☐ Lamp
☐ Reflective Blanket
☐ None needed (explain): Click here to enter text.

☐ Other (explain): rectal thermometer

19. Expected Duration of Surgery:

3-5 hours

20. Location and Size of Incision Site(s):

Scalp (3 cm)

21. Complete Description of Surgical Procedure  
(include sufficient detail that another surgeon could perform the surgery following this description):

1. Surgical instruments will autoclaved and placed on a sterile drape
2. Atropine will be administered and the rat will be allowed to wait in home cage for 10 – 20 minutes to allow the drug to take effect
3. The rat will be placed in the induction chamber that is attached to the anesthesia machine.
4. The oxygen will be turned on to 1 liter per minute and the isoflurane concentration will be set to 3%.
5. The rat will be monitored closely for signs of unconsciousness, which include lack of movement, lying on his side, a slow regular breathing.
6. When the rat is completely anesthetized, he will be taken out of the induction chamber and his nose will be placed in the anesthesia mask (attached to the stereotaxic apparatus).
7. The isoflurane will be turned down to 2%.
8. The head will be shaved from between the eyes to behind the ears and scalp cleaned with dry gauze pad.
9. The rat will be placed into the ear bars and tooth plate of the stereotaxic apparatus.
10. Lidocaine will be injected intradermally at the incision site.
11. Eye ointment and a protective eye shield will be applied.
12. To make sure that the rat is deeply anesthetized, the skin between the toes on one of the
hindpaws will be pinched firmly with hemostats.
13. If the rats shows no paw withdrawal reflex, an incision will be made down the midline of the
scalp from between the eyes to 5 mm posterior to lambda (point of meeting of the sagi-
tal and the lambdoid suture).
14. The periosteum will be gently pushed away from the bone using a cotton swab and hemostats
will be attached to the periosteum to keep the skin open during surgery.
15. The skull will be cleaned with alcohol.
16. When the skull is dry and clean, 7 – 10 small anchor screw holes will be drilled in the skull
using a high-speed micro drill with a 0.9 mm burr and screws will then be inserted. These
screws serve to anchor the implant/cannula to the skull.
17. In addition to the anchor screw holes, one large hole will then be drilled for the microdrive
according to stereotaxic coordinates using the micro drill and a 2.7 mm trephine. For AAV
surgeries, 2-4 smaller holes, one for each site, will be drilled using a 1.8 mm diameter tre-
phine.
18. Dura will be gently punctured using a 25-gauge needle and sterile saline-soaked gel foam
will be used to control the bleeding and to keep the brain surface from drying out.
19. For injection of the AAV, a micropipette will be lowered into stereotaxically-determined co-
ordinates for the nucleus eurilens, subiculum, or mpFC and pressure-injected using a sy-
ringe pump programmed to deliver the infusate at a rate of 0.1 ul per minute for 5 minutes.
After the virus infusion, the fiber stub will be lowered into place and secured in place using
dental acrylic.
20. The implant/cannula will then be lowered on top of the brain with the aid of a stereo micro-
scope to ensure that the implant/cannula does not damage the brain surface.
21. A sealant (Quik-Sil) will be applied around the implant/cannula.
22. Dental acrylic will then be applied around all of the anchor screws and the base of the im-
plant.
23. A plastic protective cone will be placed around the implant/cannula and the entire cone
wrapped in vetwrap.
24. The hemostats will be removed and the area around the incision site cleaned with Nolv-
san.
25. If necessary, sutures will be placed anterior and posterior to the implant/cannula using
standard suture material (4-0 nylon or polypropylene suture, provided by OLAM).
26. Antibiotic ointment will be applied to the incision site.
27. Banamine will be administered.
28. The rat will be taken out of the stereotaxic apparatus and taken off of the isoflurane
29. The rat will be placed in a clean cage atop a heating pad until he is active and mobile.

22. Skin Closure: Click here to enter text.

☐ Wound Clips  ☐ Surgical Tissue Glue

☐ Absorbable Suture:
  Type of suture: Click here to enter text.
  Size of suture: Click here to enter text.

☐ Non-Absorbable Suture:
  Type of suture: Click here to enter text.
  Size of suture: Click here to enter text.
23. Will Surgical Records be kept? *(Required for USDA covered species)*

- Yes
- No

24. Where will animals be housed during the recovery period?

- OLAM Surgery Suite
- OLAM Surgery/Procedure Rooms
- OLAM Animal Room (where housed)
- OLAM Lab
- Other (explain): My laboratory (147 Wolf Hall)
- Satellite Lab (explain): Click here to enter text.

25. Frequency of observation of the animals during recovery:

- Constantly
- Periodically (specify period): Click here to enter text.

26. Procedure to Maintain Normal Body Temperature during Recovery *(check all that apply):*

- Warm air or water bed
- Heat pack/pad
- Lamp
- Reflective Blanket
- None needed (explain): Click here to enter text.
- Other (explain): Click here to enter text.

**Post-Surgical Care**

**Anesthetic Recovery**
27. Procedures/Signs used to assess pain or distress:

   Analgesics and NSAID

28. Analgesic Agent(s): Banamine and ibuprofen

<table>
<thead>
<tr>
<th>Dose:</th>
<th>2.5 mg/Kg for banamine, 2 tsp. children’s ibuprofen will be mixed with water and placed in the water bottle.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Route:</td>
<td>IM</td>
</tr>
<tr>
<td>Treatment schedule:</td>
<td>Once at the beginning of surgery for banamine and 5 days postop for ibuprofen.</td>
</tr>
</tbody>
</table>

Scientific justification for not using analgesia, if applicable:

N/A

29. What is the expected time period for complete healing of surgical wounds?

5 days

30. Where will animals be housed during the healing period?

OLAM colony room

☐ Animal Room

☐ Satellite Lab (building and room number): Click here to enter text.

☐ Other (specify location and explain): Click here to enter text.

31. Specify the frequency of observation during the healing period:

1-2 times per day

32. Describe procedures for wound/incision care:

   Triple antibiotic ointment will be applied at the end of surgery and on an as-needed basis
33. Indicate when wound clips or sutures will be removed, if applicable.

N/A

### Additional Information

34. What are the anticipated outcomes of the surgery?

Rats will be able to perform working memory tasks for several weeks after recovery. The surgery allows neural activity to be monitored and manipulated during this time.

35. How long will the animals be maintained after the surgery?

1-2 months

36. Who will be responsible for post-surgical care?

The surgeon or surgeon’s assistant

37. Any additional information you wish to include:

Every surgery will have one surgeon and at least one assistant to monitor vital signs every 10 minutes while the rat is under anesthesia

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