UNIVERSITY OF CALIFORNIA SAN DIEGO

Investigating the Relationship Between Hippocampal Theta Oscillations and Firing Properties of CA1 Time Cells

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

Master of Science

in

Biology

by

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Professor Ashley Juavinett
Professor Jill Leutgeb

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The Thesis of Ameen Khan is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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2021

DEDICATION

To my mother and father

I credit all of my success to you. If it were not for your love, guidance, and support, I would have never made it this far. Thank you for everything you have done for me, I pray I only bring you happiness.

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LIST OF ABBREVIATIONS

HPC	**Hippocampus**
MTL	**Medial Temporal Lobe**
AP	**Anteroposterior**
ML	**Mediolateral**
DV	**Dorsoventral**
LFP	**Local field potential**
DG	**Dente Gyrus**
PFA	**4% Paraformaldehyde**
PBS	**Phosphate Buffered Saline**
KS test	**Kolmogorov-Smirnov Test**

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ACKNOWLEGEMENTS

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ABSTRACT OF THE THESIS

Investigating the Relationship Between Hippocampal Theta Oscillations and Firing Properties of CA1 Time Cells

by

Ameen Khan

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Professor Stefan Leutgeb, Chair

Hippocampal place and time cells are thought to play a major role in the encoding of the spatial and temporal components of episodic memory. Extensive research in rodents has been performed studying the firing properties of hippocampal place cells. However, the firing properties of more recently discovered time cells are still not well understood. In this study, we

performed electrophysiological recordings from CA1 time cells in rats as they performed a spatial working memory task on a figure-8 maze. The goal of this study was to better understand how differences in both the duration of the delay and running behavior influence the sequential activity of time cells. Time cells were recorded across two different delays of either 10 s or 30 s. The figure-8 maze was modified to contain an electric treadmill in the delay zone. The electric treadmill served to control behavior of rats during the delay zone and, depending on the extent of running, resulted in reduced theta oscillations. We found that a smaller proportion of time cells coded for later portions of the delay period. These late time cells were less stable both within and across corresponding blocks. However, when considering the entire population of time cells, CA1 time cell activity seemed to be stable between corresponding blocks irrespective of the delay length and the extent of theta oscillation during the delay period. Furthermore, the proportion of time cells did not differ depending between forced running blocks and blocks with the treadmill off, which suggests that the continuity of theta oscillations during the delay period was not necessary for the maintenance of time cell activity. Our experiment provides further insight into the mechanisms that govern the sequential activity of time cells.

INTRODUCTION

Clinical studies of amnesic and epileptic patients have long served as an indicator of the brain regions that support memory formation and retrieval. Specifically, the study of patient H.M was integral in identifying brain regions that are important for the encoding and retrieval of different types of memories, in particular of episodic memories, which require the recollection of events which occurred at a particular time and place. Patient H.M underwent a bilateral medial lobectomy (a procedure in which the complete MTL is removed) in order to alleviate and control seizures (Scoville & Milner, 1957). Post-operation, non-declarative and procedural memory remained intact in patient H.M. However, declarative and episodic memory were impaired. Patient H.M. was able to engage in conversation and retain information for up to 15 min with continuous rehearsal, but he lost memory of the previous event if his attention was diverted to a separate topic or additional information was presented. This finding suggests that a separate mechanism is responsible for the maintenance of memories over very brief compared to more separated intervals. From studies of amnesic patients such as H.M., it is generally agreed that the hippocampus and greater medial temporal lobe (MTL) play a critical role in the formation of long-term episodic memories.

Further insight into the function and underlying mechanisms governing the encoding and retrieval of episodic memories came from studying behavior common to primates in simpler animal models. Episodic memory in particular can be studied through behavioral paradigms requiring the model organism to recall what, when, and where (Clayton et al., 2001). Additionally, electrophysiology recordings from different model organisms serve as a useful tool to better understand firing patterns of individual neurons along with the overall activity of different brain regions during the performance of memory dependent tasks.

A common model organism used to study episodic memory is the rodent. Although significantly smaller in size in comparison to primates, the circuitry of the hippocampus and anatomical organization of the adjacent entorhinal, perirhinal, and postrhinal cortices is preserved in rodents (Clark & Squirea, 2013). The homology among neuronal structures of the medial entorhinal cortex in rodents and humans seems to suggest a similar function and mechanism of encoding in both species. For this reason, behavioral and lesion studies in rodents are often used to better understand mechanisms for learning and memory, which are thought to also apply to the more complex circuits in primates.

The Hippocampus is Necessary for Spatial Learning and Place Navigation

Researchers studying the function of the hippocampus performed total dorsal and ventral hippocampal lesions in rodents (Morris et al., 1982). Subjects were placed in a circular maze filled with water and learned to escape from being immersed in water by locating a platform hidden just below the surface of the water. Although rats were placed in different positions (along the circumference of the maze) at the beginning of each trial, escape latency in the control group decreased with subsequent trials. Rodents with total (dorsal and ventral) hippocampal lesions performed better on the spatial navigation task with training, yet overall escape latency remained longer across trials in comparison to control groups. The decreased performance of lesioned rodents indicated the hippocampus is integral for spatial learning.

Hippocampal Place Cells Code for Spatial Information

Electrophysiology experiments provide further evidence that the hippocampus is important for spatial learning. Researchers conducting single unit recordings found cells in the

CA1 region of the hippocampus exhibited an increased firing rate depending on the location of the rat within a closed environment (J. O'Keefe & Dostrovsky, 1971). The term "place cells" was coined for the neurons, that fired consistently at a specific location or "place field" in an environment. Collectively place cells are thought to act as or provide a spatial map of the environment.

Place Cells Fire Differentially Depending on Past and Future Locations

In addition to encoding location, place cells are thought to also code for additional stimuli. When rats performed a spatial alternation task on a T maze, place cells fired differentially depending on the previous or upcoming trial (Wood et al., 2000). In this experiment, single unit activity from CA1 neurons was recorded from rats while continuously running in an alternating pattern on a T-maze. A subset of neurons with place fields on the central stem of the maze fired exclusively during either left or right turn trials. Additionally, a separate subset of place cells fired at a significantly greater rate and/or at a different location on the central stem during left or right trials. The differential firing of place cells during the experiment was not attributable to differences in behavior, head direction, and running speed. Since the task is continuous and the rat navigated across the central stem in the beginning and end of each trial, it is unclear from this experiment whether the differential firing of place is due to the coding of future or previous trials. However, the differential firing of place cells is evidence that place cells may code for past and future location.

In a follow up study, researchers trained rats to perform a similar alternation task in a plus maze (Ferbinteanu & Shapiro, 2003). In this experiment, each trial had a definite start and end, allowing researchers to determine if differential firing of place cells was due to prospective

or retrospective coding. At the start of each trial, rats were placed randomly in one of two start locations (north or south) and travelled to one of two goal locations (east or west). Goal locations were reversed only once the rodent performed ten trials with at least ninety percent accuracy. Between trials rats were removed and placed on a waiting platform outside of the maze. Similar to the Wood et al., 2000 study researchers found place cells to be journey dependent and to fire differentially depending on the start and goal location on the maze. Since each trial had a defined start and end, researchers concluded the differential firing in start and goal locations were representative of prospective and retrospective encoding, respectively.

Place Cells Exhibit a Temporal Code and Fire at Specific Phases of Theta Oscillations

Place cells exhibit both a rate code (the increased firing rate of place cells during spatial navigation) and a temporal code. Electrophysiology studies indicate that the firing of CA1 place cells is temporally specific to different phases of theta oscillations (O'Keefe & Recce, 1993). Theta oscillations are regular sinusoidal waves in rodents that range from 7-12 Hz. In rodents, hippocampal theta oscillations are thought to indicate the active or "on-line state" of the hippocampus and are present during voluntary movement and locomotor behavior (Vanderwolf, 1969). These oscillations are representative of synaptic currents resulting from the collective firing of different neuronal structures including the hippocampus, entorhinal cortex, subicular complex, perirhinal cortices, amygdala, and cingulate cortex (Adey, 1967; Alonso & García-Austt, 1987; Leung & Borst, 1987; Mitchell & Ranck, 1980; Paré & Collins, 2000). It is believed that theta-oscillations allow for the coordinated activity between different brain regions. Theta oscillations can be recorded in all subregions of the hippocampus (Alonso & García-Austt, 1987; Brankačk et al., 1993; Winson, 1974). The manipulation and inhibition of theta oscillations leads

to deficits in hippocampus dependent behavior, suggesting their importance in the encoding and retrieval of episodic memories (Winson, 1978).

In rodents, place cells exhibit phase locking and fire consistently at a specific phase when the rodent first enters a place field (O'Keefe & Recce, 1993). Additionally, when rats progress across place fields, place cells fire earlier in phase of each theta cycle. This phenomenon is referred to as phase precession and can lead to sequential firing of place cells within a theta cycle when rodents run across adjacent/overlapping place fields (O'Keefe & Recce, 1993). Changes in spike times of place cells in relation to theta oscillations are thought to represent past, current, and future positions.

Differences in the Rate Code of CA1 Place Cells May Distinguish Between Temporally Distinct Events

In addition to coding for future and past locations during brief periods, such as a theta cycle, hippocampal cells can also code for time on much longer scales. For example, differences in firing rates and stability suggest that CA1 place cells also distinguish or code for differences in time between events that are separated by minutes and hours. When rodents navigated the same environment at different times during the day place cells were found to fire in identical locations. However, as the time interval between experiences increased, the correlation between place cell activity decreased (Mankin et al., 2012). This increasing decorrelation was found to exist for at least 30 hours. The decrease in stability of firing patterns were only observed in CA1 place cells. CA3 place cells generated consistent firing patterns across different time periods. It is hypothesized that CA1 cells receive additional input aside from CA3 neurons which allow them to code for differences in time between recurring events.

Time Cells are Sequentially Active in Working Memory Tasks

Whereas hippocampal place cells can code for time on much longer scales, hippocampal cells can also fire in sequential patterns over seconds. When cells code for time during intervals of seconds, their firing pattern is commonly referred to as time cells. Evidence that time cells may code for brief periods of time was first obtained in a study by Pastalkova et al. (2008). In this experiment single unit activity from CA1 neurons was recorded while rats performed a spatial alternation task on a figure-8 maze. During the delay period, rats ran in a running wheel. Different cells each fired for a short duration at different times in the delay period. As a population, the cells therefore appeared to fire successively or in sequence. Similar sequential firing has been observed in a working memory task for odors (MacDonald et al., 2011). In this experiment, firing patterns of time cells differed between trials with different lengths of the delay period. Altering the length of the delay period served to determine whether time cells coded for absolute intervals or the relative beginning, middle, and end delay period. Time cells were found to code for both absolute and relative time intervals. Around 40% of recorded time cells fired at the same time after the onset of the delay period. However, a majority of time cells "retimed" during trials with longer delay intervals. These cells fired at a different time periods relative to the onset of the delay period. This "retiming" of time cells was not seen immediately but after a variable number of trials. Comparisons have been drawn between the "retiming" of time cells to the altered firing patterns of place cells after the relocation of certain objects or cues in an environment.

Do Sequences of Time Cells Differ Depending on Past and Future Trajectories?

Time cells also exhibit differential firing similar to place cells (Pastalkova et al., 2008). The differential firing of both place and time cells in spatial working memory tasks suggest both types of CA1 hippocampal neurons are important in episodic and working memory. As both time cells and place cells are not necessarily two separate cell populations, it is rather unsurprising that the characteristics of time cells are similar to place cells. As described above, early evidence for differential firing was obtained by studies that did not consider sequential firing (Ferbinteanu & Shapiro, 2003; Wood et al., 2000). In these studies, place cells were found to exhibit changes in firing rates across different trials in a working memory task. From the Pastalkova et al. study, it remains unclear whether the differential firing of time cells is a reflection of the encoding for future or past events. Furthermore, the differential firing of time cells was not only limited to changes in firing rate, but also included changes in the order of sequential firing across different trials.

Time Cells Fire in Phase Relative to Hippocampal Theta Oscillations

Place cells exhibit a temporal code and fire at specific phases of theta oscillation when rats initially enter a place field. As rodents begin navigating across an environment, adjacent place cells fire in a sequential manner and individual place cells will precess and fire earlier in each following theta phase. The theta phase relationship along with the firing rate of place cells is thought to aid and improve the accuracy of place coding. Time cells in addition to place cells exhibit a similar temporal code. In the study by Pastalkova et al., although the location of rodents remained unchanged for the duration of the delay period, time cells fired earlier in phase with theta oscillations and displayed phase precession (Pastalkova et al., 2008).

Phase precession of time cells has also been observed in a recent time cell study in which rodents distinguished between different delay intervals (Shimbo et al., 2021). In this study, researchers studied the firing properties of select time cells which were stable across continuous trials. These time cells exhibited a greater average slope of phase precession during blocks of shorter delays. Additionally, these time cells fired more briefly during shorter delay trials in comparison to longer delay trials. Differences in the sparsity of firing and slope of phase precession of time cells further suggests time cells provide a scalable representation of time.

The Firing Properties of Hippocampal Cells During the Delay Interval are Inconsistent Across Different Spatial Working Memory Tasks

The observed firing properties of hippocampal cells during the delay period varies between different versions of working memory tasks. In a recent study from our lab, researchers recorded single unit activity from CA1 hippocampal neurons while rats performed a delayed spatial alternation task on a figure 8 maze (Sabariego et al., 2019). Hippocampal cells were recorded during trials with delay intervals of ten or sixty seconds. Rats performed the working memory task at above chance levels and did not show signs of any memory impairment. In contrast to the Pastalkova study, a majority of hippocampal cells were not active during either the ten or sixty second delay. Cells that were active during the delay trials did not differentiate between left and right turn trials or predict future behavior. Additionally, the sequential activity of time cells was limited to the first five seconds of each delay. This suggests that the sequential and differential firing of time cells is not necessary to bridge events discontinuous in time.

It is unclear why the firing properties of time cells differed between the Pastalkova and Sabariego experiments. In both tasks, rats perform a delayed spatial alternation task on a figure-8

maze. Some discrepancies may be due to differences in the criterion used to identify time cells. However, one of the key differences is that rats were either forced to run or were allowed to rest during the delay interval. In the study by Pastalkova et al., rats entered a running wheel and were actively moving for the duration of the delay period. In the study by Sabariego et al., rats were not actively running during the delay period and remained between two barriers on the central stem of the maze.

Based on these previous results, we hypothesized that differences in locomotor behavior during the delay period can alter the sequential activity of time cells. Hippocampal theta oscillations depend heavily on locomotor behavior and are largely absent in immobile rodents (Vanderwolf, 1969). Additionally, increases in running speed are known to increase hippocampal theta frequency and power (Sławińska & Kasicki, 1998). Along with increasing hippocampal theta frequency and power, changes in running speed and velocities of rodents have been found to increase the firing rate of place cells (McNaughton et al., 1983). Furthermore, place cells display sequential activity of time-compressed sequences within a single theta oscillation (Skaggs et al., 1996). As both place and time cells are the same neurons, it is likely that the sequential activity of both neurons arises due to a similar mechanism. If indeed theta oscillations serve as a broad temporal code and coordinate the neuronal firing of place and time cells, the differences in the extent of hippocampal theta oscillations should influence the spiking and sequential activity of these neurons.

The goal of our study was to determine whether changes in locomotor behavior of rats and the duration of the delay period of a spatial working memory task affects the firing properties of CA1 time cells. We conducted single-unit and hippocampal local field potential recordings while rats performed a delayed spatial alternation task on a modified figure-8 maze.

The maze contained a motorized treadmill which allowed for either theta or non-theta states to preferentially occur during the delay period. Our experiment provides additional insight into whether theta oscillations are necessary for the maintenance of time cells activity. It builds on previous time cell studies and provide a greater understanding of the possible function and mechanisms responsible for the sequential activity of time cells.

METHODS

Subjects

All animals used for the following experiments were approved for use by the Institutional Animal Care and Use Committee (IACUC) at the University of California San Diego (UCSD). All experiments were conducted under the guidelines outlined by the National Institutes of Health guidelines for the care and use of laboratory animals.

Behavior Apparatus (Maze Design)

A single figure-8 maze was utilized for both training and recording of rat behavior. The figure-8 maze was built using interlocking white plastic runaways that were fitted with 1 cm tall walls. The middle runway formed the stem of the maze and was 152 cm long and 14 cm wide. The left and right return arms of the maze were 10 cm wide and the same length as the central stem. The two return arms connected the reward and delay portions of the maze. Two horizontal crosspieces of 100 cm connected the central stem to the reward arms (one piece at each end). The delay site was located at the initial portion of the center arm and was 42 cm long. Inserted into the delay zone was a BIOSEB motor treadmill. The treadmill consisted of a white rolling belt which was 10 cm wide and spanned the length of the delay zone. A manual cardboard barrier and an automatic plastic barrier were utilized to control behavior within the delay zone. These two barriers prevented rats from exiting the delay zone prematurely. Rats were rewarded with chocolate flakes at the ends of the distal crosspiece prior to entering the return arms. The maze was elevated 50 cm and placed within a closed room with constant visual cues. A small light 202 cm from the maze kept the room slightly illuminated.

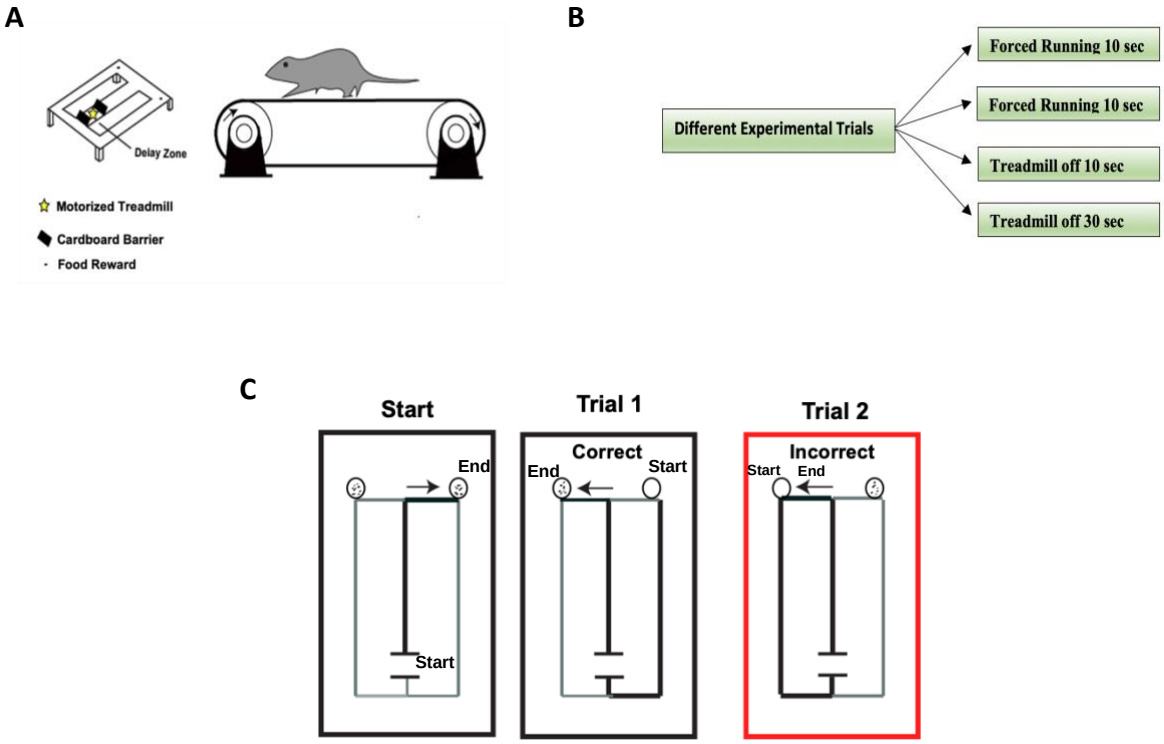


Figure 1. Figure-8 Spatial Working Memory Task. (A) Rats were trained to perform a working memory task on a figure-8 maze. A motorized treadmill in the delay zone of the maze was utilized to control behavior. (B) Rats were trained to perform in 4 different experimental conditions. First, rats were forced to run when the treadmill was on during the delay or rested in the delay period when the treadmill was off. Second, delays varied in duration and were either 10 or 30 seconds long. These two conditions were combined, which resulted in 4 types of trials. (C) To correctly perform the working memory task in any of the conditions, the reward arm that the rat travelled to had to be the opposite arm from the previous trials. If the opposite arm was chosen, a food reward was provided. Examples of a correct and an incorrect trial are shown.

Training/Habituation

In order to improve and teach the behavior to rats a habituation and shaping paradigm was utilized. Once ready for training, animals were taken to the experimental room to familiarize them with the novel environment. Additionally, animals were handled by experimenters to become more used to touch and presence of others. Prior to recording rats were trained in 4

different phases (Pretraining, Phase 1, Phase 2, Phase 3). Animals were food restricted for the duration of the training.

Prior to training, animals were weighed to monitor health and the figure-8 maze was cleaned with 70% ethanol. Cleaning of the maze improved behavior as rats were not distracted or nervous due to feces or odors from previous animals/trials.

Habituation

Chocolate cereal flakes were placed in different locations on the maze. Animals were allowed to run freely on the maze for at least 5 min. This training was repeated daily until animals explored the maze without showing any signs of fear and consistently ate the chocolate cereal flakes.

Forced Alternation (Phase 1)

To start the behavior rats were placed in the beginning of the central stem. They were forced to run in an alternating pattern and were prevented from running backwards on the figure-8 maze. They were guided on the maze manually, using cardboard barriers. Each training session consisted of 40 trials, and was repeated daily for at least two weeks.

Continuous Alternation (Phase 2)

In this stage, animals were not guided towards the reward arm containing the food reward. Rats had to recall the previous trial and run in an alternate pattern in order to receive the reward. Each training session typically consisted of 40 trials. Experimenters placed chocolate flakes in the end of one of the reward arms for each trial. Animals spent at least two weeks in

this phase or until they ran at a satisfactory rate on the maze with fewer than 10% of total incorrect trials for two out of three consecutive days.

Introduction of Delay and Treadmill (Phase 3)

Similar to phase 2, animals ran in an alternate pattern in order to receive a reward at the end of the choice arms. However, in this phase delays of 10 and 30 seconds were implemented, and the treadmill was turned on or off in the delay zone. Each training session consisted of 40 trials separated into 4 blocks of 10 trials each. Blocks were pseudorandomized and not performed in the same order on different days. However, each training session consisted of the following: 1 block with a 30 sec delay with the treadmill powered on, 1 block with a 30 sec delay with the treadmill powered off, 1 block with a 10 sec delay with the treadmill powered on and 1 block with a 10 sec delay with the treadmill powered off. This behavior was repeated daily for at least two weeks until the animal performed the behavior well and consistently. After completion of Phase 3 animals were removed from food restriction and prepared for surgery (Hyperdrive implantation).

Hyperdrive Implantation Surgery

All Hyperdrive implantation surgeries were performed using aseptic procedures. Rodents were anesthetized with isoflurane gas (0.8%– 2.0% isoflurane delivered in O2 at 1 L/min). Buprenorphine (0.02 mg/kg) was also administered as an analgesic. Animals were positioned in a Kopf stereotaxic instrument, and the incisor bar was adjusted until bregma was level with lambda. A custom built hyperdrive containing 14 tetrodes was implanted into cortical areas above the dorsal hippocampus. The implant was secured with dental cement and Metabond.

Post-operation rodents were allowed to recover, and their health was closely monitored for a minimum of five days.

Post-Surgery Retraining

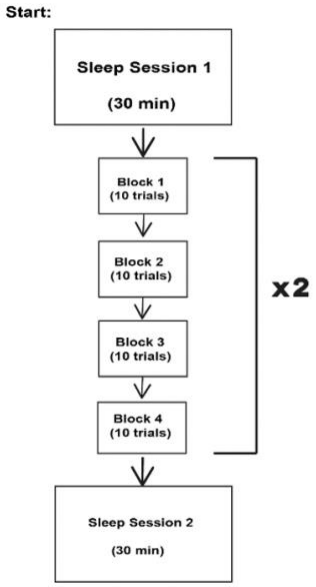
After recovering from surgery rodents were retrained on the figure-8 maze. For the first several days of retraining rodents performed a continuous spatial alternation task. Rodents typically recalled the task quickly, and after a few days of retraining, were able to complete 80 trials in a single session. After successfully performing 80 trials of the continuous alternation task over consecutive days treadmill on trials and delays were reintroduced. Neural activity and behavior were recorded after rodents consistently ran well on the treadmill and were able to complete 80 trials of the delayed spatial alternation task.

Electrophysiology Recordings

Local field potential (LFP) and single units were recorded from the CA1 region of the hippocampus using the implanted Hyperdrive. A preamplifier, tether and data acquisition system Digital Lynx SX (Neuralynx, Inc.) were used to record and collect all the data for this experiment. From each tetrode we selected a single channel to record LFP data. Out of the 14 available tetrodes, two were assigned as reference tetrodes R1and R2. Reference 1 was kept in the cortex while Reference 2 was lowered to the hippocampal cell layers (DG) to record hippocampal LFP. The acquired data was filtered at 1-450 Hz to obtain LFP and 600-6000 Hz to obtain spike data. Spikes were identified online by preset thresholds ranging from 45-50 μV. The LFP sampling frequency was set to 2000 and spike sampling frequency was 32000 Hz.

Throughout recordings, animal head direction and location were tracked using a camera and LEDs on the Hyperdrive. The camera sampling frequency was set to 30Hz.

A



B

	Block 1	Block 2	Block 3	Block 4
Recording Day 1	Treadmill off 10 sec	Treadmill off 30 sec	Forced Run 10 sec	Forced Run 30 sec
Recording Day 2	Treadmill off 30 sec	Treadmill off 10 sec	Forced Run 30 sec	Forced Run 10 sec
Recording Day 3	Forced Run 10 sec	Forced Run 30 sec	Treadmill off 10 sec	Treadmill off 30 sec
Recording Day 4	Treadmill off 10 sec	Forced Run 10 sec	Treadmill off 10 sec	Forced Run 30 sec

Figure 2. Outline of Recording Session. (A) Each recording session consisted of two sleep sessions and four blocks of trials. Recording sessions began with a sleep session in which rats were placed on an elevated platform/box and allowed to rest. After the first sleep session rats were placed on the maze to begin the working memory task. The behavior consisted of 4 different delays which were each conducted as a block of 10 trials. Blocks were repeated twice during a recording session in the same order. (B) Examples of the randomization of different delay trials into blocks across various recording days is shown on the right.

Perfusion

Following completion of recordings, rats were transcardially perfused to preserve and extract the brain. Prior to perfusion, rats were anesthetized with isoflurane gas and injected with an overdose of sodium pentobarbital. A toe/hand pinch was utilized to ensure sufficient anesthetiza. At the start of the surgery a lateral incision was made using curved blunt scissors near the diaphragm. From the initial incision, additional cuts were made contralaterally along the ribcage to expose the heart. An 18 gauge needle was carefully inserted into the left ventricle and an incision was made in the right atrium using iris scissors. The rodents were perfused with a

phosphate buffered solution followed by 4% paraformaldehyde (PFA) solution (in 0.1 M PBS). Two hours after the perfusion, brains were removed from the skull and kept in a solution of 4% paraformaldehyde for 24 h before they were transferred to a 30% sucrose solution.

Statistical Analysis and Time Cell Classification

Statistical analysis was performed using GraphPad Prism (Version 9), Microsoft Excel, and Matlab (R2018a). A One-way ANOVA was performed to test whether behavioral performance was significant between different experimental delays. A paired T-test was performed in order to compare the average theta-delta ratios in each region between forced running and treadmill off trials. A Pearson correlation of the average time maps was conducted to compare corresponding experimental blocks. A Chi-Square test was used to compare the proportion of time cells during forced running and treadmill off trials. A Kolmogorov-Smirnov test was performed to analyze the average distributions of time cells between 10 s forced running and treadmill off trials and between 30 s forced running and treadmill off trials.

Putative pyramidal cells (average firing rate on figure-8 maze between 0.1 and 10 Hz) were used in time cell classification. The stability of each neuron was calculated as a Pearson correlation of the average time map of the first 5 trials compared with the subsequent 5 trials in the same block. Cells exhibiting a stability beyond 0.3 and an average peak rate beyond 1 Hz while rats were in the delay zone were classified as a time cells.

RESULTS

First, we conducted behavioral analysis of each animal to confirm that performance of the spatial memory task was consistent between animals and across different recording sessions. Each animal on average performed at above chance levels (50%) for each of the different experimental delay trials (Fig 3). Only for a single animal, 1043, performance during 10 sec treadmill off trials was found to be significantly greater than both forced running and treadmill off 30 sec trials (pairwise t-test, Treadmill off 30 s v. Treadmill off 10 s $p<0.05$, Forced running 30 s v. Treadmill off 10 s $p<0.05$). On average rats tended to perform slightly better during shorter 10 s delay trials. However, the average performance for all animals was not found to be significantly different between the 4 different experimental conditions (One-factor Anova, $p>0.05$).

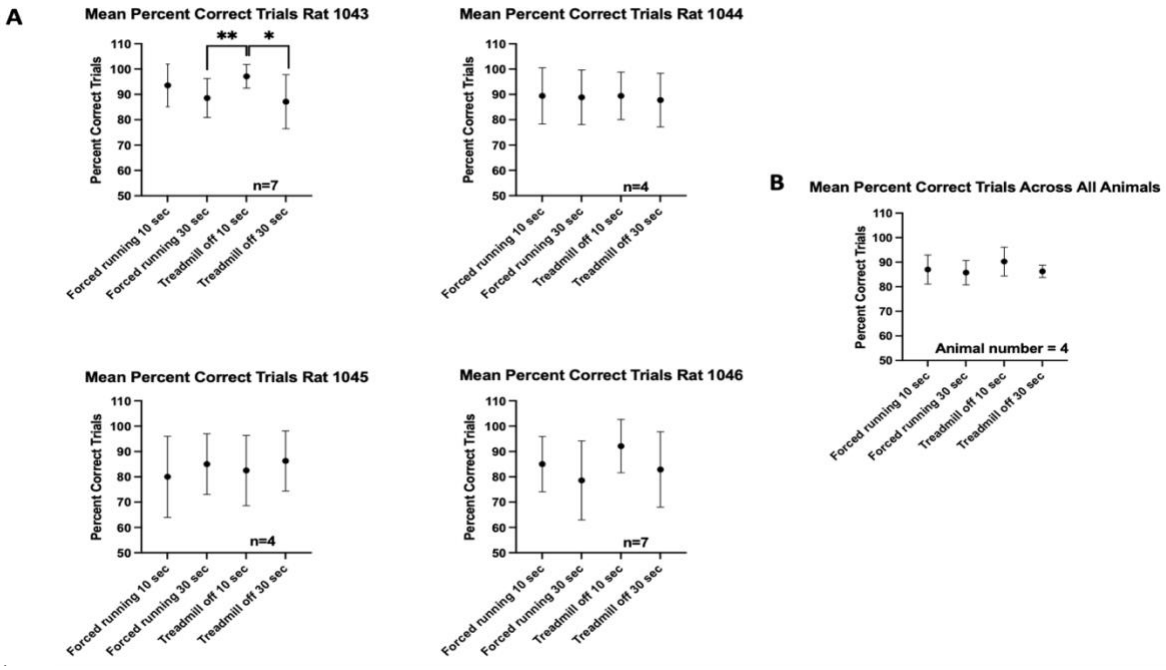


Figure 3. Behavioral Performance of Different Experimental Trials. (A) The accuracy of individual animals across 4 different delay trials. Error bars correspond to the standard deviation and n is the total number of recording sessions. (B) Average percent correct across all four animals. Error bars correspond to the standard deviation.

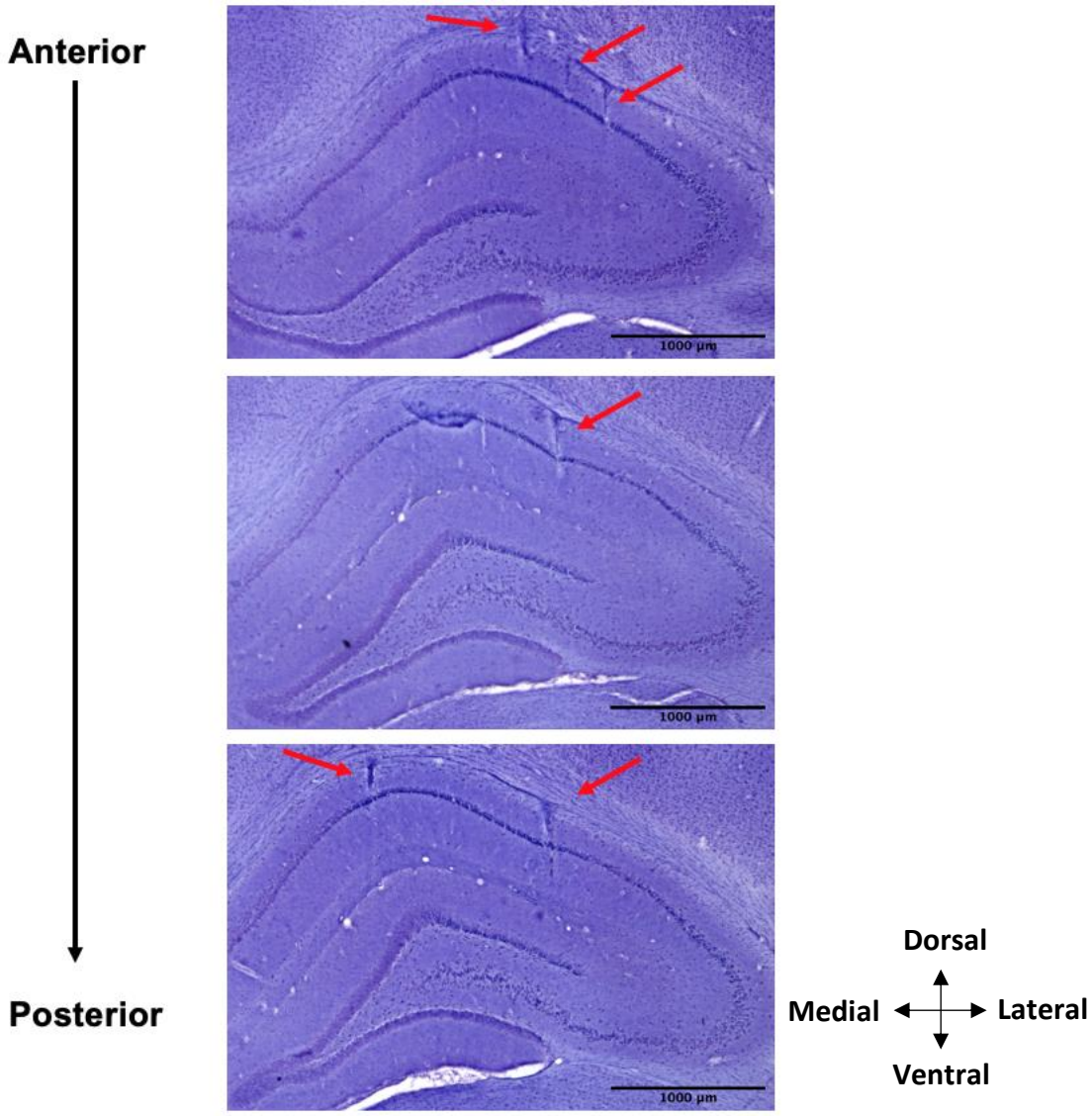


Figure 4. Tetrode Tracking Analysis. Images of coronal sections (40 µm) stained with cresyl violet. Images are organized from top to bottom with more anterior slides on top. Histological analysis confirmed that tracks (indicated by red arrows) were in the target CA1 region of the hippocampus. Scale bar is 1000 µm.

Histological Analysis Confirmed Tetrode Locations in the CA1 Layer of the Hippocampus

Tetrode tracking analysis confirmed that a majority of tetrodes were successfully positioned in the CA1 region of the hippocampus. Few tetrodes were found to be incorrectly positioned deeper in hippocampal cell layers CA3 and DG. Additionally, possibly due to tension and friction from surrounding neuronal tissue some tetrodes were found presumably stuck in the

cortex above the CA1 layer. Cells recorded from tetrodes positioned in regions outside of the CA1 layer were excluded from data analysis.

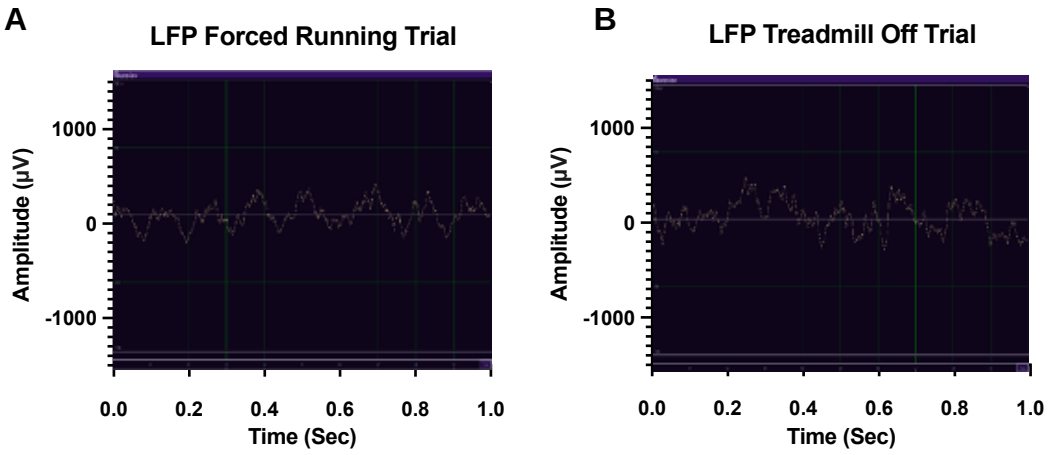


Figure 5. Raw Hippocampal LFP. Raw traces of the hippocampal LFP while rodents were confined to the delay zone of the maze. (A) Raw trace that was recorded during a delay trial in which the animal was forced to run on a motorized treadmill. (B) Raw trace that was recorded during the delay period of a resting trial in which the treadmill was not moving. Scale bar is 1 second. Prominent theta oscillations are visible when rodents are running on the treadmill. Theta oscillations are less prominent during the delay period when the treadmill is not moving and when rodents are typically more stationary and less active.

Hippocampal LFP Differed During Forced Running and Treadmill Off Trials

Tetrodes served to allow the recording of both single unit activity along with LFP from the hippocampus. Hippocampal theta oscillations were found to depend on ongoing behavior of rats during experimental trials. Weaker and less prominent theta oscillations were recorded during periods of immobility (Fig 5). More regular and powerful theta oscillations were recorded during periods of active running or when rodents were traversing different regions of the maze.

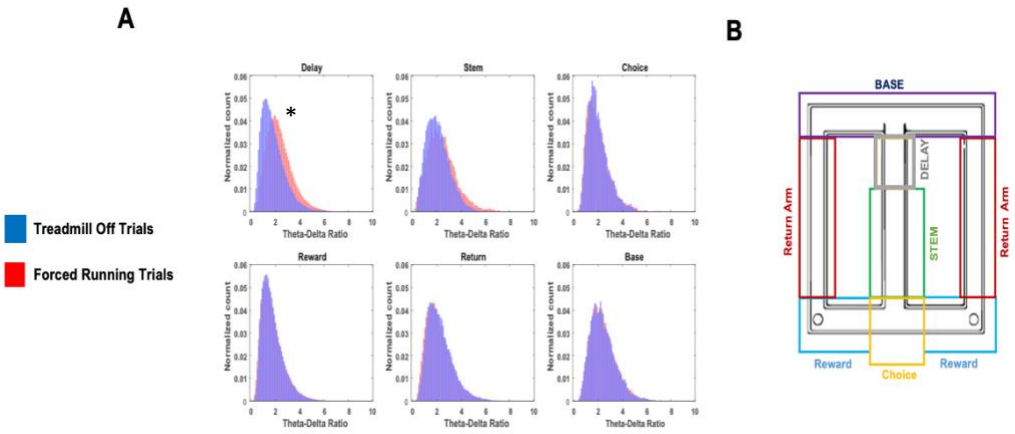


Figure 6. Comparison of Theta-Delta Ratios in Resting and Forced Running Trials. (A) Average theta-delta ratios were calculated for different regions of the maze using LFP data from multiple recording sessions. The x-axis is the theta delta ratio, and the y-axis is the normalized count. There is a visible increase in theta delta ratio in delay zone of forced running trials. (B) Different sections of the figure-8 maze are labelled.

In order to quantify differences in theta power, we calculated the average theta-delta ratio for all animals (n=4) during forced running and treadmill off trials. Theta-delta ratios were calculated separately for different regions of the figure 8 maze. The average theta-delta ratio when animals were located in the delay zone was significantly larger during forced running trials compared to treadmill off trials (pDelay <.05, pairwise t-test). Comparison of the average theta-delta ratios between forced running and treadmill-off trials for the remaining regions of the maze were not found to be significantly different (pStem=.501, pChoice=.542, pReward=0.978, pReturn=.606, pBase=0.215, pairwise t-test).

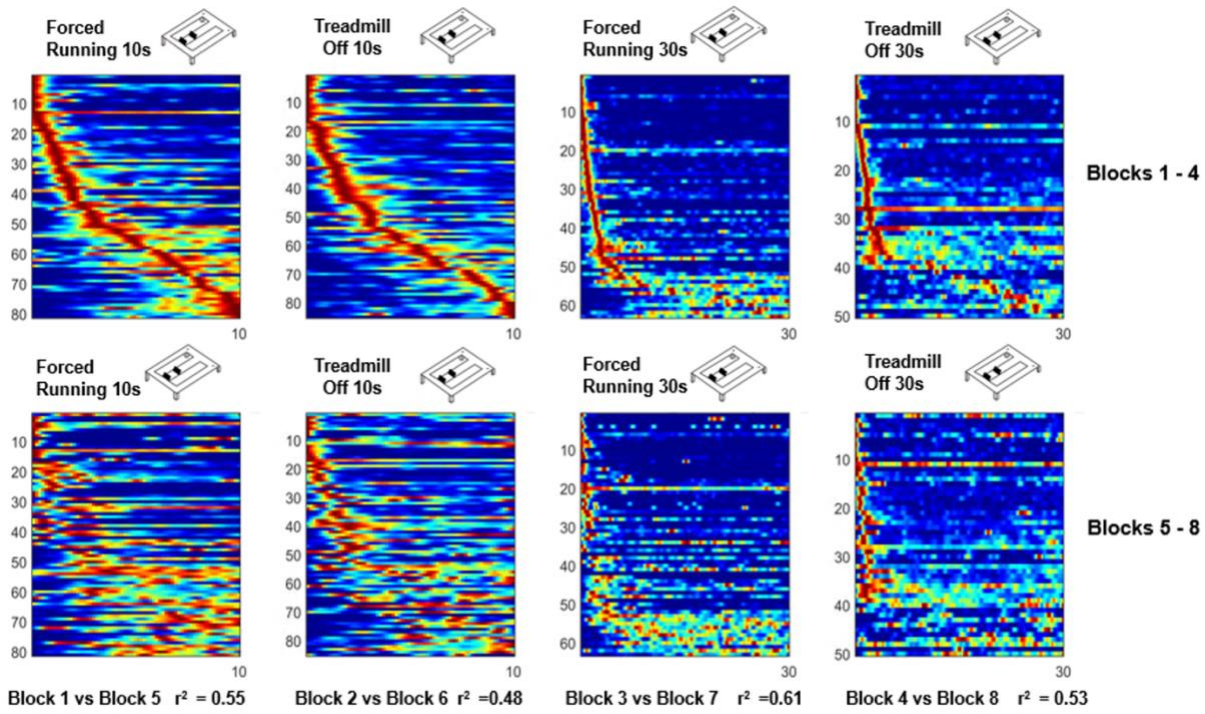


Figure 7. Sequential Activity of Time Cells. Normalized firing rate of recorded time cells during the first and second block in each of the four conditions. Cells were sorted by the latency of their peak firing rate during the first block of each experimental delay (y-axis). The x-axis is indicative of elapsed time since the onset of the delay. Correlation (r) of the sequential activity of time cells was calculated between the first block and the corresponding second block of recording sessions.

Time Cell Activity During the Delay Period does not Depend on Running Behavior

After quantifying differences in hippocampal theta oscillations across different experimental trials we performed analysis of the single unit activity of individual CA1 time cells. The peak firing rate of each time cell was measured relative to the onset of the delay period. In aggregate, time cells coded for the entire delay period (Fig. 7). A total of 282 hippocampal cells (n=4 rats) were recorded across all animals. The proportion of time cells did not differ between trial types ($p>0.5$, Chi-square test). The difference in proportion was greatest between 30 s and 10 s trials. However, these differences can be largely attributed to the criterion used to classify time cells.

Sequential Activity of Time Cells Was Stable Between Delay Intervals of Different Lengths

In order to determine whether the sequential activity of time cells is a transient effect or preserved over longer time periods, we performed an analysis between repeated blocks. When sorting time cells in the later block using the same order as in the corresponding earlier block, firing patterns in the later block retained similarity to the earlier block. This is reflected in the relatively high correlation coefficients when comparing early and later blocks. Correlation coefficients were slightly greater between forced running trials compared to treadmill off trials.

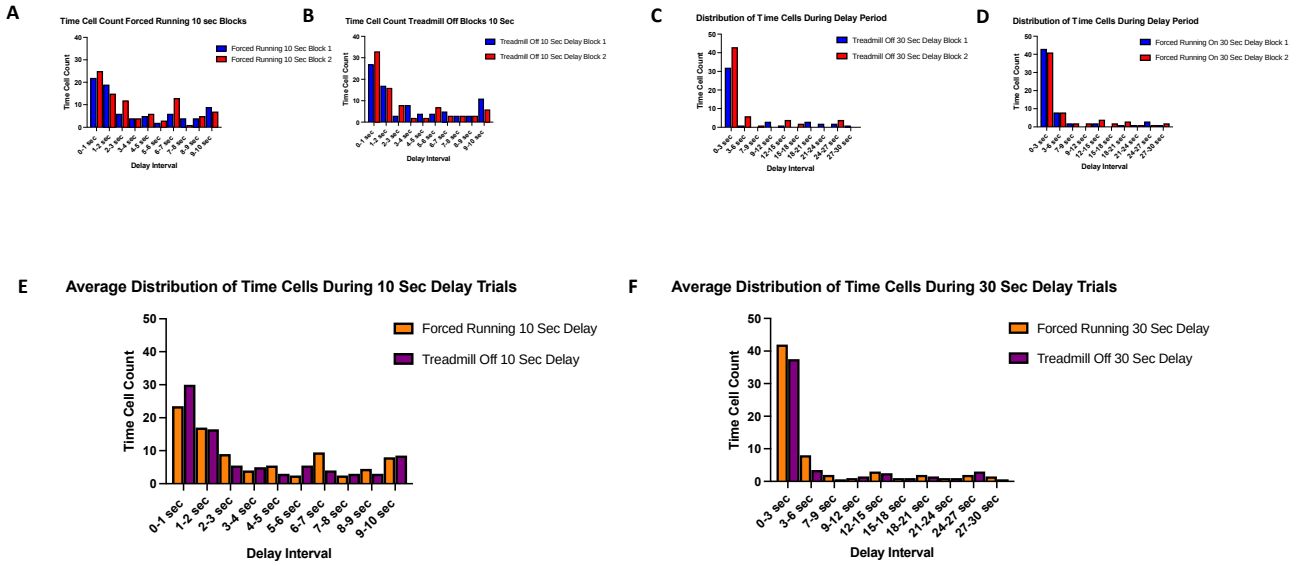


Figure 8. Time Cell Distribution Across Different Delay Trials and Blocks. (A-D) Both 10 s and 30 s delays were separated into 10 equal bins (x-axis). The first bin reflected the time immediately after the onset of the delay. For each experimental block the number of cells with a peak firing rate during each bin (time interval) was calculated. Cell counts were compared between corresponding early and late blocks of the same experimental condition. (E-F) Cell counts from corresponding blocks were averaged and compared between treadmill off and forced running trials. (KS-test forced running 10 s vs. treadmill off 10 s $p>0.95$, forced running 30 s vs. treadmill off 30 s $p>0.95$). Time cells were recorded from a total of four rats (n=4).

A Majority of Time Cells Encoded the Early Portions of the Delay Period

In all four delay conditions, time cells were observed to fire at specific intervals within the delay period. We found that the number of time cells coding for early, middle, and late

periods of the delay were not evenly distributed. We observed time cells more densely encoded earlier intervals of each delay. For each experimental condition on average, more than 40% of recorded time cells fired in the first two bins after the onset of the delay (Fig. 8). The distribution of time cells was stable and did not differ between repeated blocks of the same experimental condition. Furthermore, the average distributions of time cells during forced running and treadmill off trials were not statistically different for both 10 s and 30 s trials (KS-test, forced running 10 s vs. treadmill off 10 s $p>0.95$, forced running 30 s vs. treadmill off 30 s $p>0.95$).

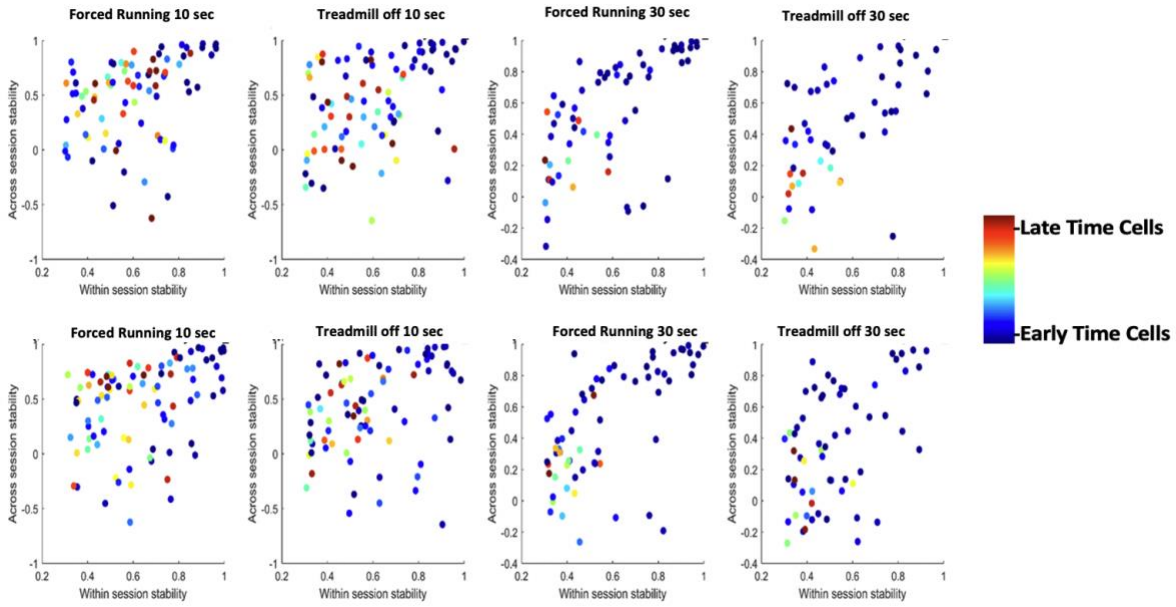


Figure 9. Within and Across Session Stability of Early and Late Firing Time Cells. Time cells were classified as early or late depending on whether they coded for earlier or later portions of the delay period. The stability of recorded time cells was calculated within a single experimental block (x-axis) and across different recording sesssions (y-axis). Colder colors (blue/green) indicate time cells which fire during earlier portions of the delay and warmer colors (red/orange) indicate time cells which fire towards the end of the delay period.

Lastly, we calculated the stability of time cells within a single block and across corresponding blocks. The stability of time cells was similar across different experimental delays. On average time cells with earlier time fields displayed greater within and across session

stability. Stability of late and early time cells was consistent between blocks and did not differ between forced running and treadmill-off conditions. However, the difference in stability between later compared earlier cells was much more pronounced in 30 s delay trials.

DISCUSSION

The sequential activation of place cells within a single hippocampal theta oscillation suggests theta rhythms play a major role in organizing the temporal firing of active neuronal ensembles. Hippocampal theta oscillations are present typically after the onset of locomotion in rodents, and the frequency and amplitude of hippocampal theta waves have been observed to be proportional to the speed of movement (Bender et al., 2015). Properties of time cells have been found to differ between working memory tasks in which rodents were either actively running or allowed to rest during periods of delay. Time cells were reported to differentiate between left and right trials and predict future behavior during 10 s delay periods in rats actively running in a running wheel (Pastalkova et al., 2008). However, when rats were not actively running during the delay period, time cells did not predict accuracy of future behavior or differentiate between left and right trials (Sabariego et al., 2019). Furthermore, in the Sabariego et al. study, time cells were observed to only code for the first ~5 s of the delay and consistent sequential activity was not observed during longer 60 sec delay trials. In our study we controlled the behavior of rats during the delay period of a spatial working memory task to induce either strong or weak hippocampal theta oscillations. As expected, we observed more prominent theta oscillations during forced running trials in which rats were actively running in place. Behavioral performance in the spatial alternation task was not affected by introduction of the electric treadmill. Across all animals the average performance did not significantly differ between different experimental conditions ($p>.05$). Irrespective of behavior during the delay interval, time cell activity was found across two blocks of trials that were ~20 minutes apart, and the temporal coding patterns seemed stable across corresponding experimental conditions (delay length and treadmill on/off). A majority of time cells were found to code for earlier intervals immediately after the onset of

the delay. Time cells coding for later delay intervals displayed less stability both between consecutive trials and between blocks that were separated over longer intervals (~tens of minutes). Overall, the proportion of time cells in each experimental condition was not significantly different ($p>0.5$) suggesting that running behavior and high-amplitude hippocampal theta oscillations are not necessary for the maintenance of CA1 time cell activity.

Increased Running Behavior Induced Powerful Theta Oscillations

As hypothesized by previous studies, during periods of increased theta activity the hippocampus is likely receiving an increase in cholinergic and GABAergic input from the medial septum (Hasselmo et al., 2002; Marrosu et al., 1995). This increased input from the medial septum then leads to the coordinated activity of hippocampal neurons across spatial and temporal scales. In our study, hippocampal LFP was found to display a strong correlation with behavior. In trials during which the treadmill was powered on, rats were forced to run for the duration of the delay period. During these forced running trials, strong theta oscillations were observed in the raw LFP recording. Theta oscillations were not prominent during the delay period of treadmill-off trials during which rats were not actively running and were allowed to rest. This finding was expected as hippocampal theta oscillations have been reported to increase in both frequency and power with increased velocity and to be largely absent in immobile rodents (Sławińska & Kasicki, 1998; Vanderwolf, 1969).

To quantify the differences in Hippocampal LFP we calculated the average theta-delta ratios between different regions of the figure-8 maze. In awake rats hippocampal CA1 delta power was inversely correlated with locomotion and velocity (Schultheiss et al., 2020). Delta oscillations increase when rats are stationary for even brief moments. Therefore, theta-delta

ratios serve as a useful metric to characterize and compare rat behavior between different regions of the figure-8 maze and different experimental conditions.

In our study, the average theta-delta ratios were significantly different in the delay zone when trials with forced running were compared to treadmill off trials ($p<.05$). Average theta-delta ratios were not significantly different between remaining regions of the maze ($p>.05$). These results were expected and affirm because behavior of rats differed only in the delay zone while rats were either forced to run or allowed to rest on the treadmill. Behavior in remaining regions of the maze remained similar between forced running and treadmill off trials.

Increasing Delay Duration Resulted in Only Minor Reduction of Working Memory Performance

Working memory requires the maintenance and manipulation of recently acquired sensory information. Performance of a spatial alternation task decreases in rats with the introduction of a delay (Ainge et al., 2007). Furthermore, increasing the delay length from 2 s to 10 s led to further decline in performance. In this study, both the duration of the delay period and activity of rodents differed across experimental conditions. Differences in running behavior during the delay period did not affect the ability of rats to perform the spatial alternation (i.e., average accuracy and performance of the spatial working memory task did not differ in treadmill off and forced running trials, $p>0.05$). However, similar to the Ainge et al's study, we observed a lower average working memory performance during longer delays, although these differences in performance were not found to be significant in our small cohort of rats ($p>.05$).

Temporal Representation is Not Different Across Corresponding Blocks

A unique feature of our recording paradigm is that it afforded us the opportunity to measure the stability of time cells across both consecutive trials and between separated corresponding blocks. On average time cells displayed consistent sequential activity within repeated trials and between corresponding blocks. This suggested time cells were not exclusively a transient phenomenon, but that temporal coding of similar time points within the delay intervals was consistent across longer time periods.

Increased Proportion of Time Cells are Active During the Beginning of the Delay

The proportion of time cells active during different intervals of the delay period was not similar at each moment in time. In both forced running trials and treadmill-off trials a majority of time cells were active within the first two seconds after the onset of the delay period . Fewer cells coded for later segments of the delay. The distribution of time cells during the delay period was not statistically different between treadmill off and forced running trials (see Fig. 8). Our results were consistent with findings from previous studies that reported prominent sequence coding only for the 5-10 seconds of a 60 sec delay (Sabariego et al., 2019).

Additionally, the decreased stability and number of time cells coding for later portions of delay period indicates successful performance of the alternation task is likely not dependent on the continuous sequential activity of hippocampal time cells. Instead, memory retention and retrieval may be supported using separate inputs and structures from the medial temporal lobe. Working memory impairments are significant in MEC lesioned rats and have been found to increase in trials with longer delay periods (Sabariego et al., 2019). Although CA1 time cell activity may play a greater role in retrieval and performance during shorter delay periods, MEC

inputs may play a more critical role in the performance during longer delay trials. One hypothesis is that time cells are encoding scalar time. If this were the case, the decreased stability of late time cells suggests that the estimation of elapsed time decreases significantly shortly after the onset of the delay.

Time Cell Activity Is Not Dependent On Hippocampal Theta Oscillations

Time cells have been observed to bridge discontinuous events in several different working memory tasks. In a majority of time cell studies, rodents were actively running for the duration of the delay period. Fewer time cells have been observed in studies in which rats were resting or not actively running during the delay period. The inconsistent observations between different time cell studies suggests that the sequential activity of time cells is dependent on running behavior and the onset of powerful hippocampal theta oscillations. In our study we recorded a total of 282 hippocampal pyramidal neurons. The same proportion of time cells were observed during both forced running and treadmill off trials ($p>0.5$). The similar sequential firing along with Pearson's correlation coefficient between corresponding early and later blocks suggest that sequential activity of time cells was not exclusively a transient phenomenon, but temporal coding of similar events was consistent across longer time intervals. From our study, we found that the strength of theta oscillations did not determine the extent of the maintenance of time cell activity.

Although it appears maintenance of time cell activity may be independent of the extent of theta oscillations during the delay interval, it is possible the generation of theta oscillations during forced running trials is necessary for the initial generation of time cells. In a follow up experiment, we will include a separate control group which performs only treadmill off trials. A

lack of time cell activity in the control group would suggest that consistent theta oscillations during the delay period are indeed required for the generation of time cells.

An alternative hypotheses is that time cells can be generated independent of hippocampal theta oscillations. For example, they may be generated by computations in other brain structures. The frontal cortex and striatum are known to play a role in interval timing during working memory tasks (Lustig et al., 2005), and cortical neurons may persist or continue to represent/retrieve a particular stimuli for an extended time period. If firing of these neurons is in an oscillatory manner, the striatum may be able to discern temporal components of the delay from the phase of the cortical oscillations. It is possible, that this temporal information is then signaled downstream to time cells within the hippocampus. This could be tested in future studies that lesion or inactivate these brain regions while recording from hippocampal neurons. Additionally, temporal coding by the hippocampus may not be dependent on hippocampal theta oscillations. A separate synfire chain model has been proposed to explain the reactivation of the same spatiotemporal pattern of cortical cell populations (Nunez, 1983). In this model, sequential activity arises through a layered feed-forward network model in which activity is propagated from one cell population to another. However, detection of synfire chain activity is limited by current recording technology, as detection of such neural networks likely requires the simultaneous recording from hundreds of individual neurons (Gerstein et al., 2012).

CONCLUSION

The discovery of both hippocampal place and time cells has suggested that the hippocampus may code for both the spatial and temporal elements of different episodic memories. Although the firing properties of place cells are well studied, the firing properties of time cells are still unclear. In our study we found that during both 10 s and 30 s delay trials the maintenance of sequential activity was not dependent on increased locomotor or running behavior during the delay period. As expected, the increased velocity of rodents during the delay period led to an increase in the average power of hippocampal theta oscillations within the delay zone of the maze. Prominent hippocampal theta oscillations were not necessary for the maintenance of time cell activity and the average distribution of time cell activity remained the same irrespective of differences in behavior and theta power. Overall a majority of cells were found to have a peak firing rate within the first two (of 10) bins of each delay. As a general trend, cells which encoded earlier intervals/bins tended to display both greater stability between consecutive trials and between corresponding blocks. The decreased proportion of time cells encoding later intervals of 30 s delay periods along with the decreased stability of late time cells suggests time cell function was not critical for successful performance of the alternation task in longer delay intervals.

From our experiment, the exact function of hippocampal time cells still remains unclear. However, this study serves to provide additional insight into the different neuronal mechanisms responsible for the maintenance of the sequential firing of hippocampal time cells during working memory tasks.

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