UNIVERSITY OF CALIFORNIA SAN DIEGO

Novel Behavioral Paradigm for Investigating Temporal Pattern Separation in Mice

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

In

Biology

by

Emily Yan

Committee in Charge:

Professor Matthew Shtrahman, Chair
Professor Brenda Bloodgood, Co-Chair
Professor Pamela Reinagel

The Thesis of Emily Yan is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

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ABSTRACT OF THE THESIS

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Master of Science in Biology

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A region of the mammalian brain called the hippocampus is crucial for memory formation. However, how the hippocampus plays a key role in encoding time-varying information is not fully understood. The hippocampus is unique because it is one of only two brain regions in mice that generate new neurons throughout life in a process

known as adult neurogenesis. This peculiar anatomical feature has been hypothesized to be central to the ability of the hippocampus to make fine distinctions between similar experiences in a process known as pattern separation. New hippocampal neurons, known as newborn dentate granule cells (DGCs), are believed to play a vital role in the brain's ability to perform pattern separation. However, DGCs rarely fire action potentials, so traditional electrophysiological techniques that depend on data collected from highly active neurons are not optimal and cannot reliably identify the rarely-firing DGCs. As a result, there are significant gaps in knowledge about how newborn DGCs contribute to the ability of the hippocampus to perform pattern separation, particularly on time-varying information. Central to this study is the optimization of a behavioral paradigm in which head-fixed mice perform a novel temporal pattern separation task that is amenable to study with two-photon calcium imaging. This study establishes a novel training protocol to allow for in-depth study of the temporal aspects of pattern separation in vivo.

Chapter 1. Introduction

Temporal Pattern Separation

Even though we experience the world as a continuous stream of information, our hippocampus is often confronted with the decision of whether to create new distinct memories (in a process known as pattern separation) or to generalize information from similar experiences (in a process known as pattern completion). An often-overlooked aspect of memory formation is the question of how the hippocampus processes time-varying information during experience to create discrete memories. It remains unclear how a specific region of the hippocampus called the dentate gyrus (DG) integrates time-varying streams of information, or temporal patterns, from everyday experiences into discrete memories.

Unique aspects of hippocampal anatomy serve as the basis for many theories of memory formation. Pattern separation is believed to be performed by Dentate Granule Cells (DGCs) and other cells located in the DG and underlying hilus of the hippocampus (Drew et. al 2013; Yassa and Stark 2011). There are three main regions of the hippocampus: DG, CA3, and CA1. Sensory information from our experiences is funneled through layer II of the entorhinal cortex (EC) through axonal projections known as the perforant path to the DG. From the DG, information is passed along to CA3 via mossy fibers. Then, CA3 projects to CA1, completing the tri-synaptic pathway (Deng 2013). Although this tri-synaptic pathway is the most well-known in the hippocampus, other circuits have been identified. For instance, a parallel pathway known as the excitatory monosynaptic pathway flows from EC (layer III) to CA1 (“temporoammonic pathway”)

and back to EC (layer V) (Nakashiba 2018). In addition, there is evidence for a 'backprojection' from CA3 to the DG (Scharfman 2007) . However, significant gaps in knowledge remain regarding how this complex hippocampal circuitry optimizes learning and memory.

The DG has approximately four to five times more neurons than its input or output regions, the EC and CA3, respectively (Aimone et al. 2011; Deng et al. 2010). As a result, the large network of DGCs is tasked with processing input from relatively fewer cells and generating a condensed output. Although there are more neurons in the DG, they fire action potentials very rarely. Conversely, neurons in the EC are highly excitable and fire action potentials frequently (Yassa and Stark 2011). Many computational studies postulate that the DG performs pattern separation by amplifying differences between similar, yet distinct inputs (Bakker 2008). These computational models propose that the DG creates a sparse representation of its EC inputs which it then sends to CA3 (Rolls 2006).

It is postulated that the DG plays a key role in initial information processing (Scharfman 2007). It is believed that a few DGCs fire in a uniquely sparse pattern that is specific to each experience (Johnston et al. 2016). Thus, similar yet distinct experiences that may not have been represented as distinctively in the EC have much more pronounced differences in neural representation in the DG. As such, if one were to be exposed to two very similar experiences, the EC would represent these experiences using very similar patterns of high neural activity (Johnston et al. 2016). The brain's ability to perform pattern separation is crucial to avoid memory interference and confusion when

one is tasked with recalling similar information (Colgin 2008). The DG's unique ability to perform pattern separation can distinguish experiences that may have substantial overlap and enhance memory recall ability (Neunuebel and Knierim 2014; Kyle 2015).

Evidence for Hippocampal Role in Pattern Separation

The hypothesis that the DG is critical for pattern separation is supported by accumulating evidence from human studies. For instance, patients with damage to their hippocampus were found to have reduced pattern separation ability (Kirwan 2012). Electrophysiological studies in humans with damage to their hippocampus have shown that the hippocampus is crucial for detecting and responding to novel auditory stimuli (Knight 1996). Moreover, when human subjects performed a pattern separation task involving the encoding of pictures of common objects, researchers observed that the DG had decreased activity when patients were presented with familiar objects (e.g. pictures of objects that were repeatedly presented) and had increased activity when presented with novel objects (e.g. pictures of objects which are similar but different from the familiar objects) (Bakker et al. 2008). However, a major limitation of these studies is that fMRI techniques detect global changes in hippocampal activity, whereas subtle changes in neural activity between hippocampal subregions cannot be reliably identified. As such, significant gaps in knowledge remain regarding how the DG performs pattern separation on the cellular level.

The auditory 'oddball' paradigm is a test used in humans used to examine physiological responses to novelty, and many animal studies suggest a role for the hippocampus in auditory 'oddball' detection (Ruusuvirta 2013). Some animal studies

have used contextual conditioning to analyze pattern separation (Deng 2010; Yassa and Stark 2011). In one experiment, rats freely explored enclosures that gradually transformed from being square in shape to circular. Then after using electrophysiological techniques to record from the DG, the researchers observed that firing patterns in the DG, and not in any other hippocampal area, changed significantly when the walls of the enclosure were changed slightly. Additionally, mice with impaired function of the DGCs were unable to differentiate between similar contexts during contextual fear conditioning (McHugh et al. 2007). These animal studies support the hypothesis that the DG carries out pattern separation to distinguish between similar but distinct experiences. However, these behavioral tasks are not suitable for studying the temporal aspects of pattern separation.

Adult Neurogenesis

The DG is a unique region in the brain where new neurons are formed during adulthood in a process known as adult neurogenesis (Deng et al. 2010; Eriksson et al. 1998). The majority of DGCs are present beginning in early developmental stages and do not divide later in life (Tonegawa et al. 2012). However, the remainder of DGCs (adult-born neurons) develop from precursor stem cells throughout adulthood (Tonegawa et al. 2012). Morphologically, newborn DGCs initially form dendrites projecting to the molecular layer and then send axons to CA3. Newborn DGCs also exhibit increased dendritic branching compared to their mature counterparts (Goncalves 2016). Within four to seven weeks, the adult-born DGCs are fully integrated into the surrounding hippocampal network (Zhao et al. 2006; Deng 2010; Kempermann 2004).

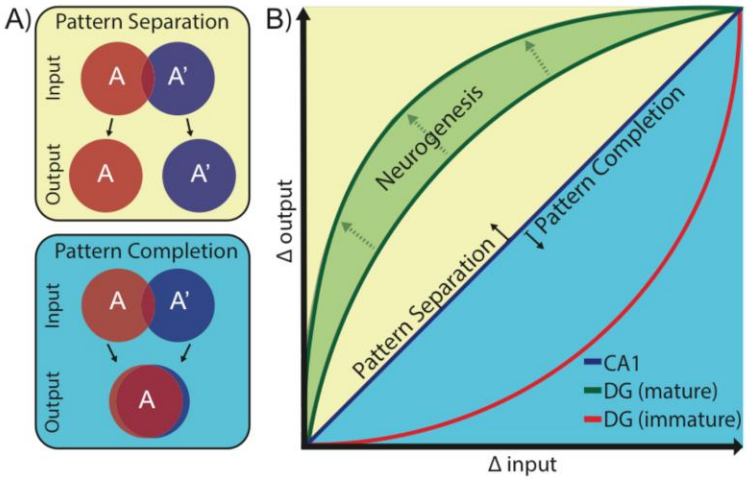

Figure 1. Pattern separation ability of the hippocampus. A. Similar yet distinct experiences, A and A', are uniquely represented by distinct neuronal representations in pattern separation. However, pattern completion is shown as similar neuronal inputs (A and A') are represented by similar neuronal outputs. B. Immature DGCs, although broadly tuned to input stimuli, are believed to increase the ability of the hippocampus to perform pattern separation. Adult neurogenesis that occurs in the DG of the hippocampus is also believed to help facilitate its ability to pattern separate. This schematic is adapted from Yassa and Stark and Johnston et al.

Since immature DGCs have lower activation thresholds compared to mature DGCs, it is likely that EC inputs activate immature DGCs at a higher probability compared to mature DGCs. As a result, this heterogeneous population of cells in the DG supports the idea that this area of the hippocampus contributes to pattern separation (Marin-Burgin et al. 2012). Notably, the heightened plasticity of immature DGCs may allow them to uniquely contribute to the ability of the DG to perform pattern separation. Pattern separation studies in humans have shown a decline in performance as the rate of adult neurogenesis in the DG decreases with age (Stark and Yassa 2010). These findings suggest that immature adult-born DGCs may be necessary for the DG to perform pattern

separation (Stark and Yassa 2010). However, little is known about how mature and newborn DGCs process time-varying information during behavioral pattern separation.

Pattern Separation Task for Mice

In our experiment, we aimed to understand how the DG handles time-varying information from similar experiences to form distinct memories. A mechanistic explanation for how the DG performs temporal pattern separation remains elusive due in part to two significant limitations. First, there are substantial obstacles associated with observing the activity of DGCs in awake behaving animals. Limitations in electrophysiological recording methods of cells in vivo are unsuitable for investigating the activity of DGCs. The uniquely sparse activity of DGCs further complicates the use of statistical methods for spike sorting. Moreover, current techniques for identifying cell types remain unreliable for identifying mature and immature DGCs using characteristics such as subtle differences in their activity. Until recently, in vivo imaging techniques have not been able to measure activity of the granule cell layer, due to its depth in the mouse brain (Goncalves et al. 2016).

Secondly, current studies of pattern separation often utilize behavioral task, such as contextual fear conditioning, are not suitable for probing temporal aspects of pattern separation. Moreover, they are not compatible with head-fixation. Considering these challenges, we have designed a novel pattern separation task which allows for two-photon calcium imaging of DGCs in awake behaving mice. Using these new techniques, we can investigate the unique contributions that mature and immature DGCs make towards the process of temporal pattern separation.

We designed this experiment around a phenomenon that frequently occurs in our everyday lives: a familiar tune that may jog a memory. Tones and songs with varying familiarity serve as useful temporal patterns to study pattern separation and pattern completion. There is evidence that leads us to believe that the hippocampus is involved in our ability to detect novelty and deviance in auditory tasks (Rutihauser 2006). As such, we have designed a temporal pattern separation task for head-fixed mice to learn a temporal sequence of auditory tones. Head-fixed mice were trained to recognize a three- or four-note template song, and we tested their ability to discriminate between the familiar template song and random non-template songs. Additionally, mice were trained using two left-right lick ports such that behaviors and rewards were matched for each side. We can use this novel task to overcome these challenges and investigate the temporal components of pattern separation.

Two-Photon Imaging

Two-photon imaging has significant deep tissue imaging advantages over traditional microscopy techniques. Traditional techniques are limited to shallow imaging depths around 100μm before light scattering at greater depths renders images unusable. Novel two-photon excitation uses two photons that arrive simultaneously with enough combined energy to excite a fluorescent molecule. One key advantage of two-photon imaging is that the use of two-photons allows for near-infrared light excitation that allows for unparalleled imaging of deep brain structures (So et al. 2000).

For our experiment, two-photon imaging reduces light scattering which minimizes noise and improves clarity of the image (Helmchen 2005). Moreover, new red-shifted

Ca^{2+} indicators (jRGECO1a) allow for greater sensitivity and ability to observe neural activity (Dana 2014). We also utilize a custom-built goniometer system which allows for fine-tuning of the cranial window tilt to decrease aberration and increase two-photon excitation efficiency (Arimoto 2004). As a result, we can image more than 700um deep into the hippocampus with 75 to 110mW of excitation energy (Horton 2013).

Chapter 2. Materials and Methods

Temporal Pattern Separation Task Training

The aim of the pattern separation task was to train mice to recognize and differentiate between whether the presented song is the familiar template song or a similar but distinct non-template song. Mice were trained to lick a lick port on their right side to receive water reward in response the template song; conversely, they were trained to lick a lick port on their left side to receive a water reward (Kool-Aid, 4 uL) in response to a non-template song. The deviations of non-template songs from the template songs were calculated in half-steps on the chromatic scale (Figure 2). During each trial, the mice had a 2-second lick window immediately following the end of the song to make a decision (Figure 3B). If a mouse licked outside of the lick window or did not lick at all, the trial was regarded as 'no-lick' trial.

After undergoing headbar surgery, seven-week old C57BL/6 mice were acclimated to experimenter handling for two days prior to being introduced to the experimental setup. Seven-week old F1 hybrid mice (C57BL/6 mice crossed with ICR/HaJ mice) were also used in this experiment. Once mice were comfortable with human handling, they were acclimated to the self-paced cylindrical treadmill and allowed to become familiarized with the light-tight and sound-proofed boxes. Head-fixation of the mice on this treadmill setup was first performed in 5-minute sessions to minimize stress on the animal. When mice no longer showed signs of stress while being head-fixed on the experimental setup, they were trained in a total of five different phases with the aid of water deprivation to maintain 85% of their baseline body weight (Guo 2014). Mouse

weights were tracked every day of the week. Mice that did not adequately maintain their weight received supplemental water by hand as-needed. Additionally, mice that did not receive a minimum of 1mL of water during training days also received supplemental water to obtain the required daily amount of water.

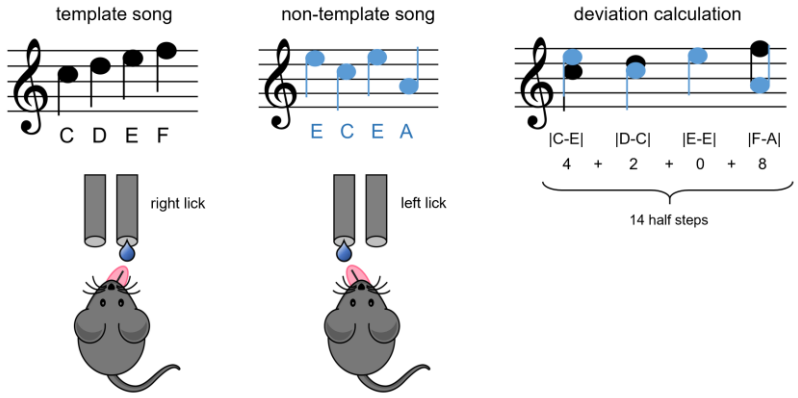

Figure 2. Temporal pattern separation task for head-fixed mice. Left: Mice are trained to lick the right lick-port for a familiar temporal sequence of auditory tones, a template song, and to lick the left lick-port for any songs that deviate from the template song, a non-template song. Right: An example calculation for deviation calculation is shown. For each trial, each non-template song's deviation from the template song was calculated in half steps (hs) on the chromatic scale.

Songs consisted of four 300-ms long pure tones played sequentially with a 200ms pause between tones. Tones ranged from 8 to 10 KHz, which is in the optimal hearing sensitivity range for these C57BL/6 mice. In addition, this frequency range is less susceptible to age-related hearing loss in the higher frequency ranges (Ison 2007). The mice were trained to recognize a four-note template song. Non-template songs were generated randomly from a pre-determined set of 17 possible notes. Licking for template and non-template songs was registered through left-right lick-ports that were conductive. The measure of deviation of non-template songs from the template song as well as left and right licks for each trial were observed in real-time and recorded (Figure 4).

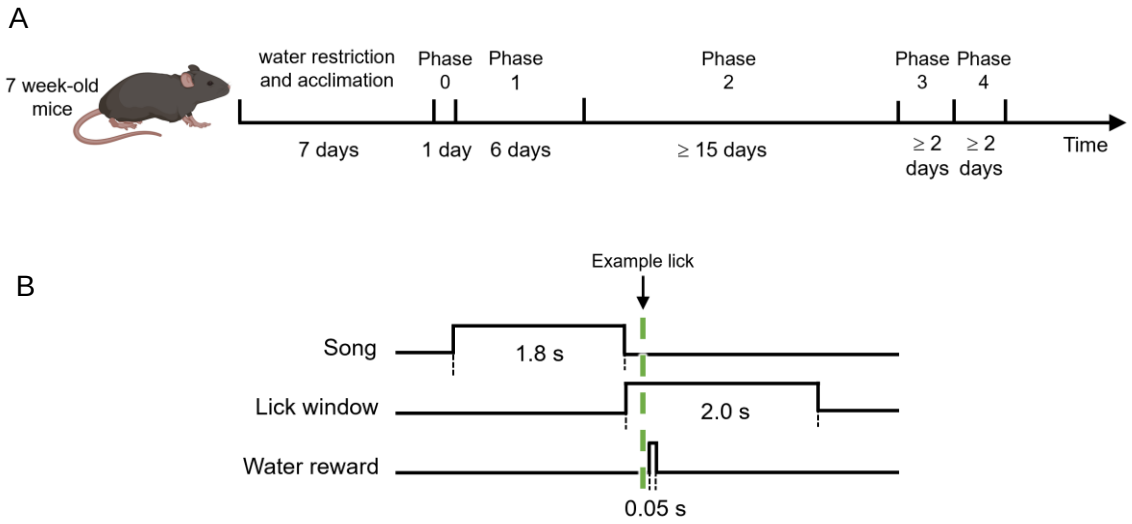

Figure 3. Temporal pattern separation task and individual trial event timeline. A. Timeline showing the sequence of events of the temporal pattern separation task training. **B.** Each individual trial consisted of a 1.8-second long sequence of auditory tones that was immediately followed by a 2-second window during which mouse licks were registered. Water reward was given immediately following a correct decision.

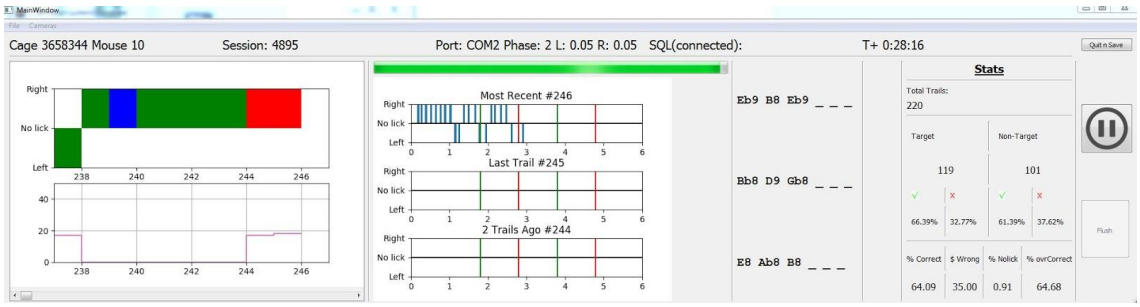

Figure 4. Custom-built software for observing real-time mouse performance. Mice performing the pattern separation task were observed during training. The software indicated whether template or non-template songs were presented and when individual mouse licks were recorded. Additionally, certain metrics were tracked and displayed in real-time such as correctness (green), incorrectness (red), and encouragement drops received (blue).

The temporal pattern separation task training consisted of a total of five training phases, Phase 0 through Phase 4, that the mice completed sequentially. During Phase 0,

mice received block training in which they were presented with three-song blocks of the one song type, either template or non-template, and these blocks alternated automatically. This phase was unique because mice were given water reward automatically at the end of each trial. The goal of this phase was to teach the mice that water reward was available through both the left and right lick ports following the presentation of each respective song type. Once mice had exhibited licking behavior during Phase 0, usually following one day of training, they would be advanced to subsequent training phases (Table 1). Mice were advanced to the more difficult phases after they had performed at or above 70% correctness for two days in a row. Phase 1 is very similar to Phase 0, except that the mice only receive water reward when they lick correctly. Phase 2 is when bias breaking is implemented. Phase 2 has a unique block-based bias breaking design whereas Phase 3 has a single-song-based bias breaking design (Figure 5).

Table 1. Temporal pattern separation task phases.

Phase #	Block training	Bias breaking	Reward drops
Phase 0	Yes	No	Automatic
Phase 1	Yes	No	Earned
Phase 2	Yes	Yes (phase-specific)	
Phase 3	No	Yes (phase-specific)	
Phase 4	No	No	

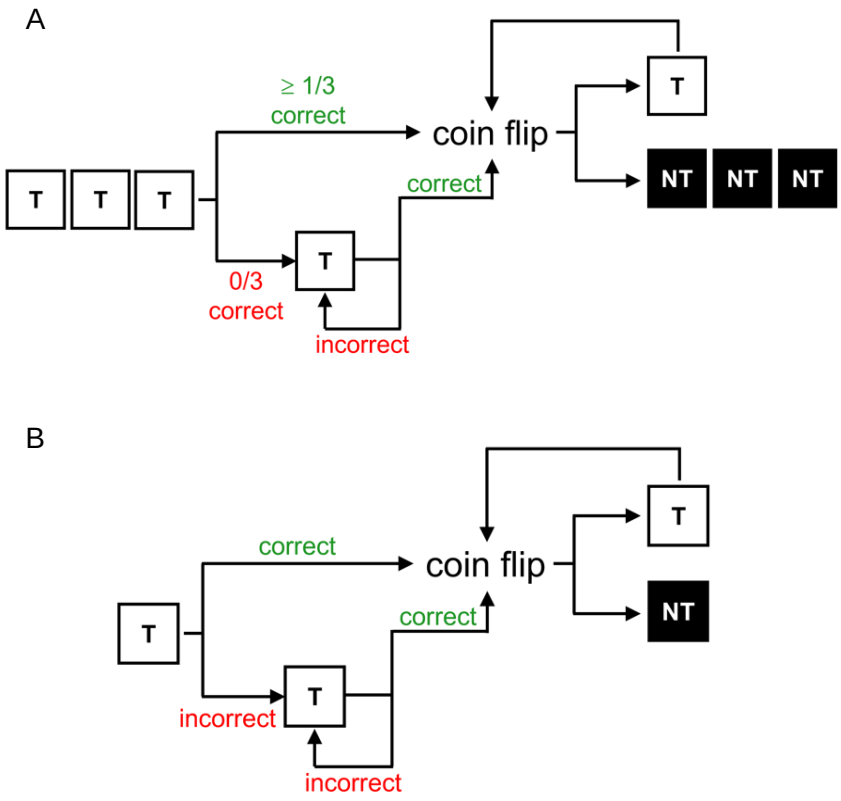

Figure 5. Temporal pattern separation task bias breaking design. A. Diagram of bias breaking design for Phase 2 of task training which involves block training. Template (T) and non-template (NT) songs are shown. Decisions are ultimately guided to be determined randomly by a coin flip (random 50/50 chance). **B.** Diagram of bias breaking design for Phase 3 of task training, which does not include block training. Template (T) and non-template (NT) songs are shown. Similarly, decisions are eventually determined randomly by a coin flip (random 50/50 chance).

Mouse Headbar Surgery

In preparation for the head bar surgery, mice were anesthetized with isoflurane. To prepare the area for surgery, the mouse's scalp was shaved with electric clippers. The mouse's head was then immobilized using a stereotaxic surgery apparatus (Stoelting, Wood Dale, IL). Throughout the duration of the surgery, the mouse's body temperature was maintained at 37°C with a small electric heating pad. Additionally, eye ointment was

applied to the eyes to maintain their health during surgery. Then, the mouse scalp was treated with Betadine and alcohol, in an alternating fashion, three times. A scalpel was used to make a midline incision to expose the skull. The skull was cleaned and dried using sterile cotton-tipped applicators. Orthodontic acrylic (Lang Dental Mfg., Wheeling, IL) was used to affix a titanium head bar to allow for reliable head-fixation of the mouse for task training. After surgery, mice were transferred to a recovery cage before they were returned to their home cages.

Motorization of Treadmills and Mouse Housing

Mice were trained using both self-paced and motorized cylindrical treadmills. To motorize treadmills, brushless motors were custom-fitted to the axels of the cylindrical foam treadmills. The motors were used during the inter-trial intervals, between the conclusion of the lick window and beginning of the first tone of the next song. Motor speeds were increased incrementally during training, up to a maximum speed of 0.16 m/s, only when mice did not show signs of distress or discomfort when the motorized treadmills were running.

Mouse housing was varied during the experiment. Some mice were assigned to be housed with their littermates in their home cage while others were assigned to be housed in an enriched environment enclosure. Up to 4 mice were housed in each enriched environment. Each EE cage had 3 running wheels, 3 huts, and many tunnels.

Viral Labeling of Dentate Granule Cells and Chronic Window Surgery

In this experiment, we use an adeno-associated virus (AAV) vector to deliver a red Ca^{2+} indicator (jRGECO1a) that is driven under the excitatory cell-specific promoter

(AAV.CAMKII.jRGECO1a, Salk GT3 Core) to visualize activity in DGCs in awake behaving animals. Mice were first anesthetized with isoflurane. Then, the head was immobilized using a stereotax. After shaving the scalp with electric clippers, a midline incision was made with a scalpel to expose the skull. A dental drill was used to drill a small hole (<1mm in diameter) over the somatosensory or motor cortex. A Nanoject injector (Drummond Scientific) was used to inject 1uL of the virus. Following the surgery, the mouse scalp is closed using cyanoacrylate adhesive (VetBond), and the mouse was allowed to recover from anesthesia before it was returned to its home cage.

In preparation for two-photon imaging of the hippocampus, cranial window implantation and viral labeling of dentate granule cells were performed. These additional surgical procedures were selectively preformed on mice that demonstrated expert performance following pattern separation task training. A craniotomy was performed using a dental drill to remove a circular portion of skull that was 3 mm in diameter, directly over the right hemisphere of the brain. The cortex overlying the hippocampus was removed by aspirating using a blunt tipped needle and vacuum line. Sterile gel foam soaked in saline was used to help stop bleeding throughout this process. Then a 3 mm glass coverslip set into a titanium window implant, 3mm in diameter and 1.7mm deep, was implanted into the craniotomy. This chronic window implant was attached using dental cement. Immediately after surgery, buprenorphine (0.1mg/kg, s.q.) and carprofen (5mg/kg, i.p.) were administered to the animal to relieve pain and inflammation. After recovering from surgery in a recovery bed, the mice were then allowed returned to their home cages.

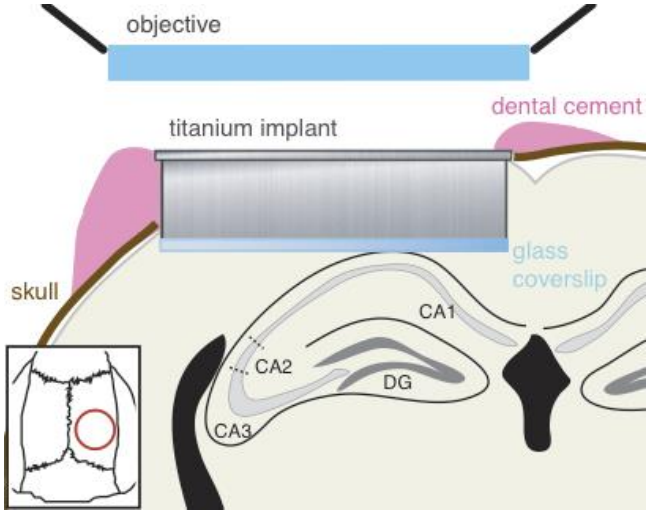

Figure 6. Chronic window implant for imaging of the mouse DG. A chronic titanium implant with a 3mm glass coverslip is surgically placed to allow for deep two-photon imaging of the DG. This implant surgery was adapted from Gonçalves et al. 2016.

Two-photon Imaging of the Hippocampus

Mice were trained and imaged using a two-photon laser scanning microscope (Movable Objective Microscope; Sutter Instruments) with a femtosecond-pulsed laser (Chameleon Ultra II, Coherent) with a water immersion objective (0.8 NA, Nikon). Images were taken for populations of DGCs in the mouse DG that expressed jRGECO1a. During video acquisition, mouse behavior performance data were recorded.

Chapter 3. Results

Preliminary Mouse Performance

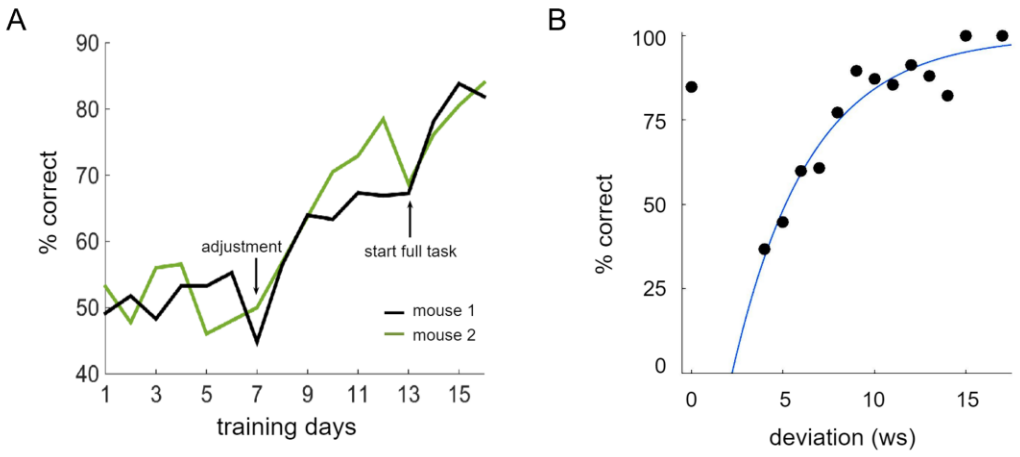

Figure 7. Mice successfully learning a pattern separation task in a 15-day period. A. Preliminary data is shown of mice trained to recognize a four-note template song, C D E F. Learning curves of the two best performers are plotted. A training adjustment was made on day 7 whereby bias breaking was introduced to eliminate lick port bias. Full task marks the beginning of Phase 3. **B.** The performance of one mouse is plotted as a function of non-template song deviation from the template song. A deviation of 0 indicates the template song. Mouse performance from the last three training days (days 14 through 16) is plotted. A line of best fit is plotted in blue.

During preliminary tests of the temporal pattern separation task, we were able to train mice can successfully within a 15-day time frame (Figure 7). This time frame is important because memories formed in the hippocampus are eventually transferred to the cortex of the brain after a two-month period (McKenzie and Eichenbaum 2011). Thus, the training timeline was optimized to ensure the hippocampal dependency of this memory task. Although the time frame and mechanism by which this occurs is not completely understood, memories formed in rodents are believed to be more dependent

on the hippocampus earlier in life (McKenzie and Eichenbaum 2011). As such, younger 8-week old mice were used in our experiments.

Initially, the mice were trained using block training where 3 template songs were played, followed by 3 non-template songs, and so on. However, it was observed that many mice preferred to lick on one side more than the other. To eliminate this lick port side bias, a training adjustment was made on day 7 in the form of bias breaking implementation into the task software. If a mouse did not correctly identify at least one song within a 3-song block of either template or non-template songs, the same block was repeated until the mouse licked at least once on correct side for that 3-song block (Figure 5). Mouse performance improved steadily following this bias-breaking adjustment which encouraged them to pay attention to both lick ports. Overall, these initial mice performed the task with >75% accuracy within 15 training days (Figure 7).

Optimization of the Temporal Pattern Separation Task

We were hopeful that the duration of task training could be shortened for hippocampal dependency of the task and two-photon imaging capabilities. Since the initial 6 days of Phase 1 training resulted in a mice having a strong bias and task performance around chance-levels, we reasoned that it may be possible to circumvent Phase 1 to expedite training. However, upon removing Phase 1, the majority of mice were not able to achieve at least 2 days in a row of 70% correctness in order to advance to Phase 2. Without Phase 1 training, these mice often did not reach expert-levels of performance (i.e. Phase 3 or beyond) and did not lick as frequently (Figure 8). With these

findings, we began training all mice with 6 days of Phase 1 so that they could receive ample reinforcement and continue to be motivated during training.

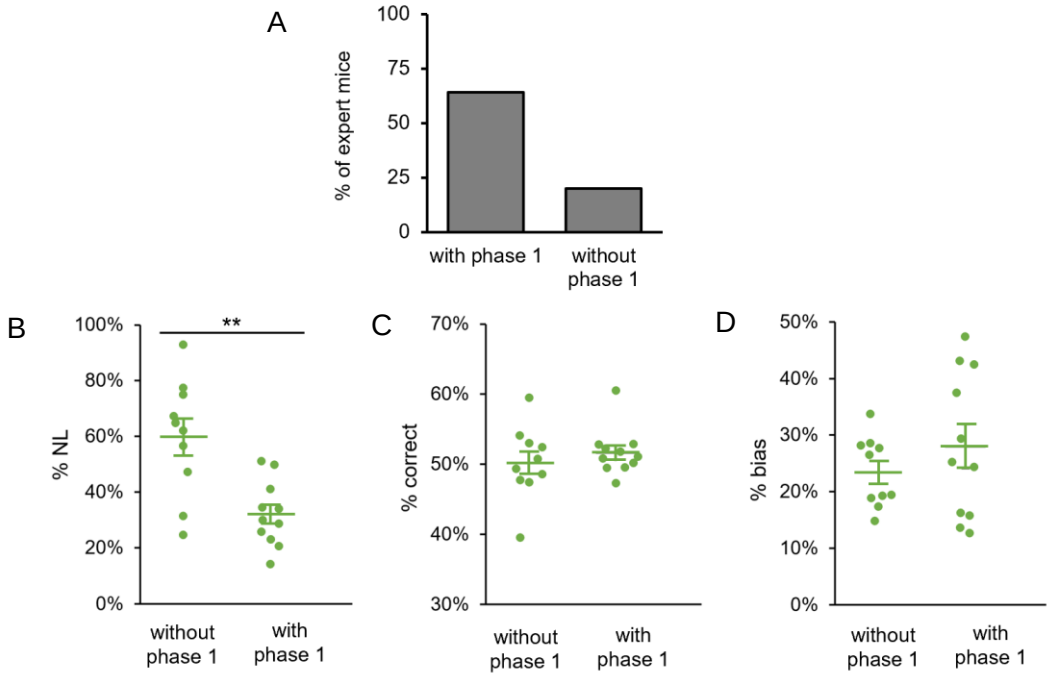

Figure 8. Phase 1 is beneficial for learning. A. Data is shown from several mice that were trained with and without phase 1. Expert performers are mice that progressed to Phase 3 or beyond. **B.** The percentage of trials that mice did not lick is shown for the first 6 days of training for mice that received Phase 1 and mice that received Phase 2. Mann-Whitney U Test (**p < .001) **C.** The percentage of trials that mice licked correctly is shown for the first 6 days of training for mice that received Phase 1 and mice that received Phase 2. **D.** The degree of biased lick-port preference is shown for the first 6 days of training for mice that received Phase 1 and mice that received Phase 2.

We began to explore other avenues to optimize training. We often observed heterogeneity in moue performance, even between littermates that were trained on the same template song. We were inspired by a recent experiment by Albergaria et al. in which they were able to reduce variability and expedite learning for mice trained on a behavior task by implementing motorized treadmills. While effect was present in both slow (0.1 m/s) and fast (0.3 m/s) conditions, learning occurred faster in the fast motor

condition. (Albergaria et al. 2018). Unfortunately inclusion of motorized treadmills, set to around 0.16 m/s, did not make a difference in the variability of mouse performance or time of training when compared to mice trained using self-paced treadmills (Figure 9).

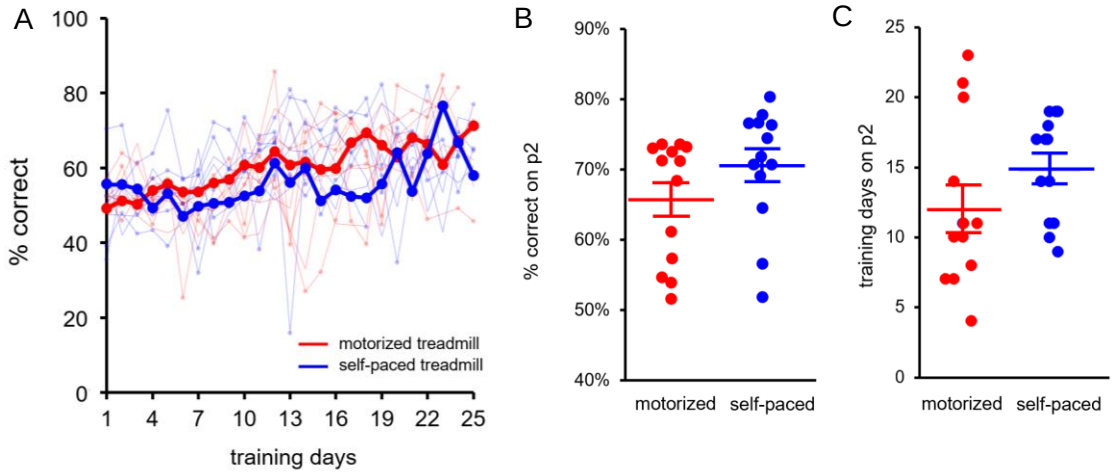

Figure 9. Motorized treadmills do not decrease variability or improve learning. A. The average percent of correct trials is plotted for 13 mice trained with motorized treadmills (green) and 12 mice trained with self-paced treadmills (blue). **B.** Average percent correct on the three best-performing days of phase 2 is plotted for mice trained with motorized treadmills (left) and without motorized treadmills (right). Data represent the mean ± SEM. **C.** Number of training days on phase 2 is plotted for mice trained with motorized treadmills (left) and without motorized treadmills (right). Data represent the mean ± SEM.

Next, we explored the possible learning benefits of housing mice in an enriched environment. Studies in humans indicate that greater rates of exercise are associated with increased hippocampal size and signs of increased rates of adult neurogenesis in the DG (Berchtold 2002, Cotman 2002, Erickson 2011). Some studies have shown that mice with access to running wheels have greater spatial pattern separation ability (Creer 2009). Additionally, there is evidence that animals with access to running wheels have improved learning and increased neurogenesis (van Praag 2005). Four mice at most were housed in each enriched environment enclosure. The enriched environments consisted of 3 running

wheels and numerous huts and tunnels for the mice to run and explore when they were not training. Unfortunately, the mice housed in the enriched environment performed at similar levels on the task as mice housed in their home cage (Figure 10).

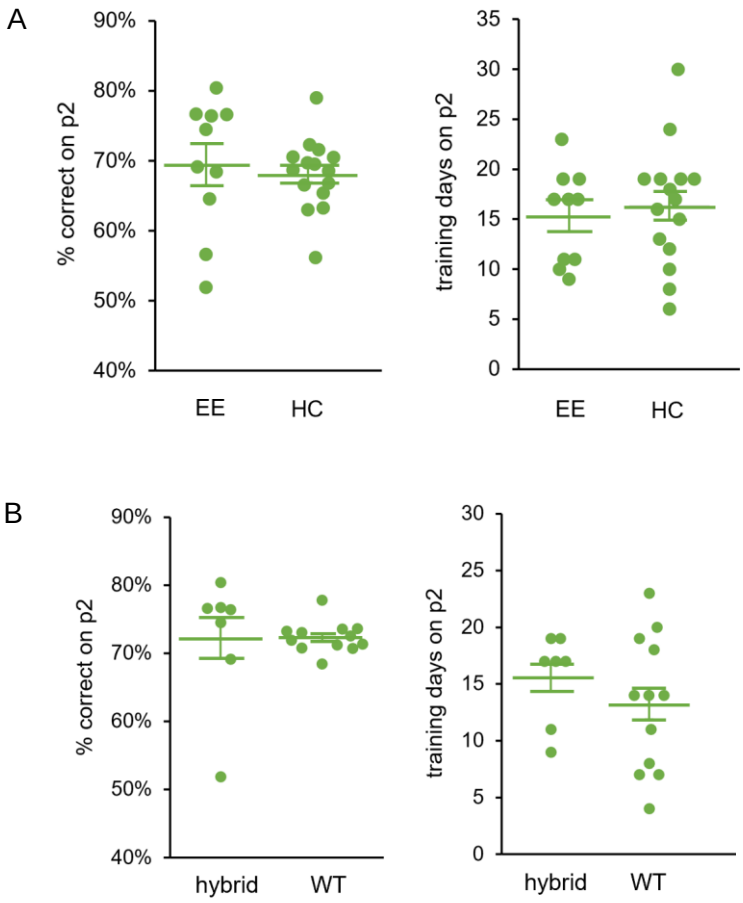

Figure 10. Animal housing and genetic background do not affect performance. A. Left: Average percent correct on the three best-performing days of phase 2 is plotted for mice housed in an enriched environment and in a home cage. Right: Number of training days on phase 2 is plotted for mice with housed in an enriched environment and in a home cage. **B.** Left: Average percent correct on the three best-performing days of phase 2 is plotted for mice with a hybrid genetic background and wild-type. Right: Number of training days on phase 2 is plotted for mice with a hybrid genetic background and wild-type. Data represent the mean ± SEM.

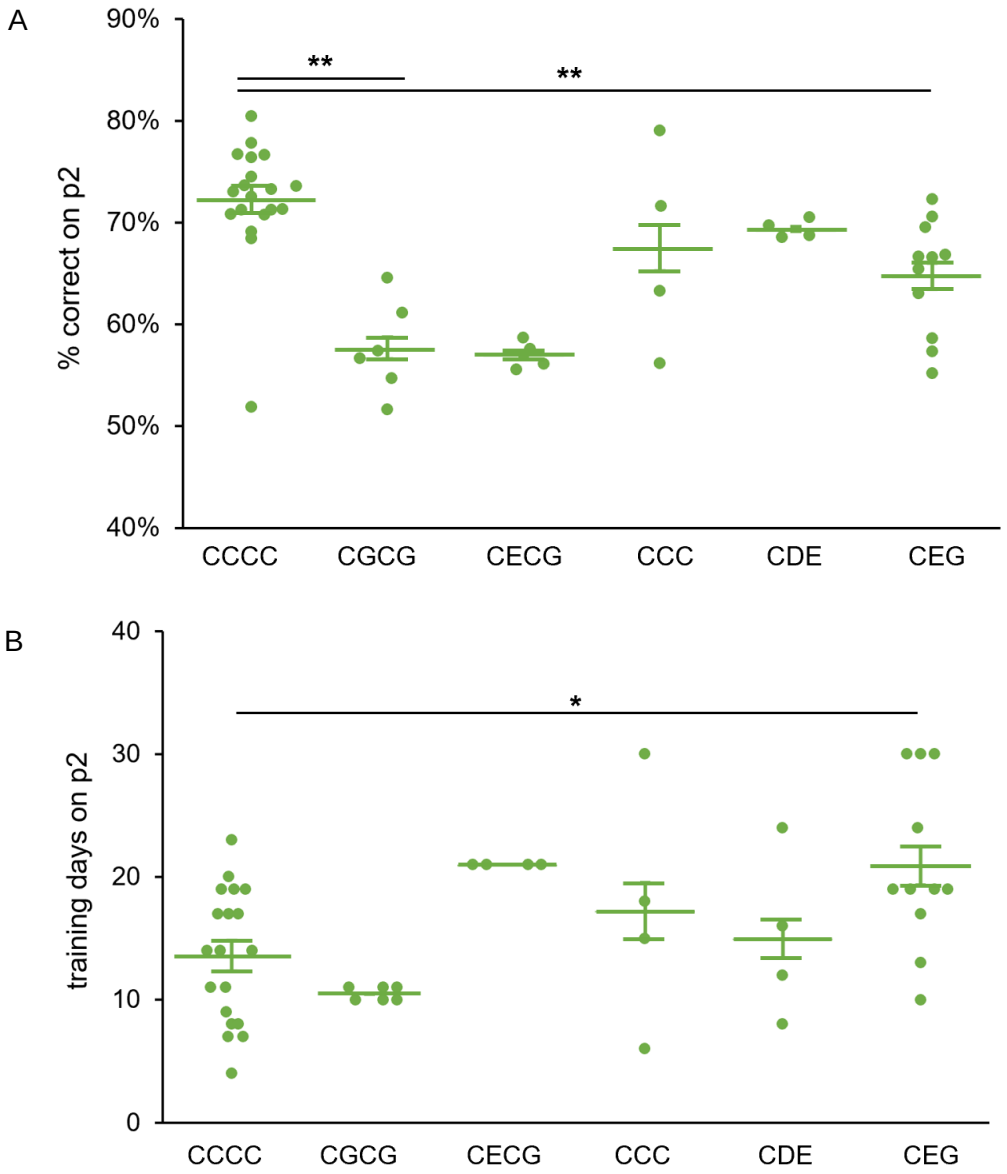

Figure 11. Repeating 4-note and 3-note template songs are learned more effectively. **A.** Average correctness is plotted for mice that were trained with various 4-note and 3-note template songs. Data represent the mean ± SEM. Mann-Whitney U Test (**$p < 0.01$). **B.** Training days on Phase 2 is plotted for mice that were trained with various 4-note and 3-note template songs. Data represent the mean ± SEM. Mann-Whitney U Test (*$p < 0.05$).

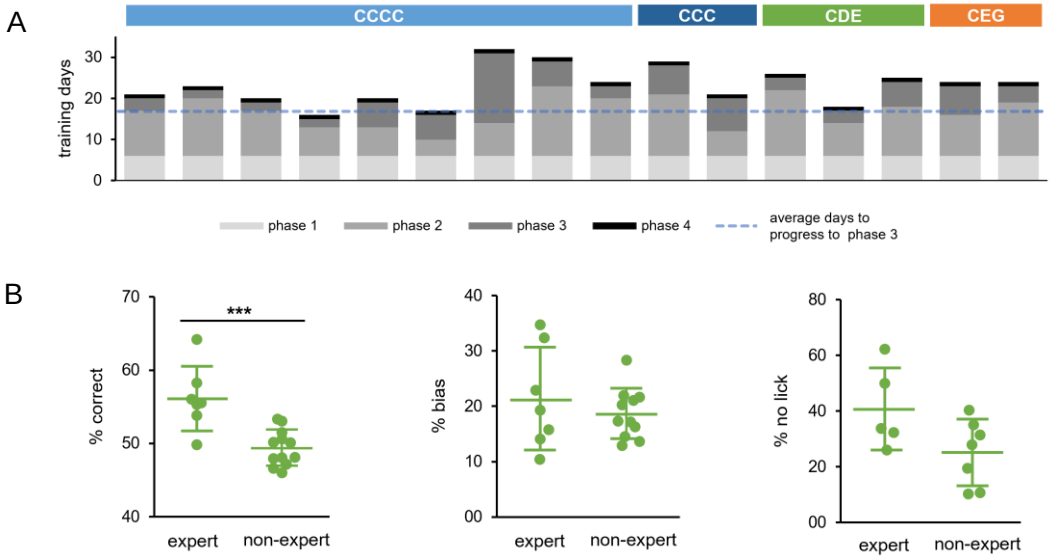

Figure 12. Correctness can be used to identify expert performers early on. A. Phase progression from phase 1 to phase 4 is shown for expert mice. Template song is indicated. Expert mice require 16.8 ± 0.9 training days to reach Phase 3. **B.** % correct (left), % bias (middle), and % no lick (right) are shown for expert and non-expert mice during the first 6 days of Phase 2 training. Data represent the mean ± SD. (***p < 0.001)

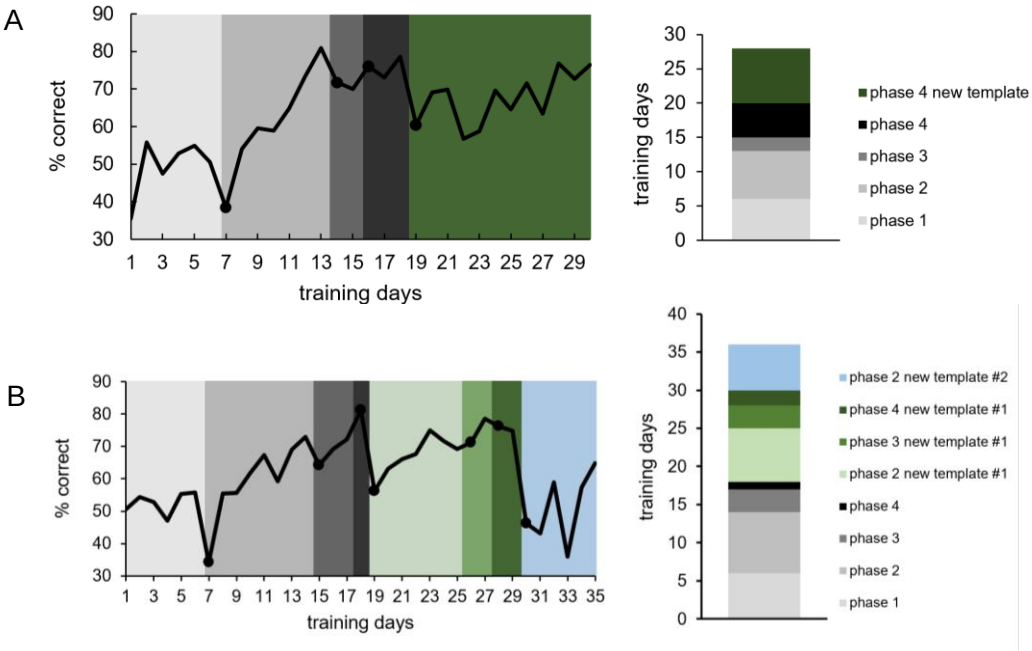

Figure 13. Expert performers can learn new template songs. A. Left: Correctness for one mouse is plotted over time. Right: Phase progression is shown. Its first template songs were CCCC then GCGC. **B.** Left: Correctness for one mouse is plotted over time. Right: Phase progression is shown. Its template songs were CDE, EAbB, and BFG.

We then explored whether the genetic makeup of the mice (C57BL/6J), which is highly inbred strain, was a limiting factor for mouse performance on the task. We crossed ICR/HaJ mice with C57BL/6J mice to produce a hybrid line and tested their performance on the task. The two types of mice with different genetic backgrounds performed at similar levels of average correctness (Figure 10). After unsuccessful attempts to optimize behavior training by modifying the training treadmills, housing, and genetic makeup of mice, we began altering the template song in hopes of decreasing the task difficulty. After experimenting with various 4-note and 3-note template songs, it became apparent that 4-note template songs with repeated notes and 3-note template songs were easiest for the mice to learn (Figure 11). In addition, it seems that we can identify expert performers early on by assessing their performance during the first six days of Phase 2 (Figure 12).

With this finding, we began testing if expert performers on the task could be trained on an ‘easier’ template song and switched to a new, more difficult template song. Mice who advanced to Phase 4 of an ‘easier’ template song were able to progress to a new, more difficult, template song (Figure 13). This method of behavior training has proven most promising for investigating temporal pattern separation in mice.

Chapter 4. Discussion

Our task is a improvement from contextual conditioning tasks traditionally used to assess pattern separation ability in animals as it allow researchers to study the temporal aspects of pattern separation. Because animals trained using contextual conditioning are freely-moving, imaging of hippocampal neurons is not a viable option for investigating how the DG performs pattern separation in vivo (Yassa and Stark 2011). Therefore, our novel behavior task allows head-fixed mice to perform a pattern separation task while being imaged using two-photon microscopy.

Temporal Pattern Separation Task Optimization

In this experiment, our greatest hurdle was finding the best way to train mice most efficiently for the purposes of probing the temporal aspects of pattern separation. We found that it may be the case that mice require a minimum of three weeks for them to become comfortable with the behavioral setup and learn the contingencies related to the task (i.e. running on the wheel, becoming conditioned to the sounds, smells, and lick ports in this new environment). Additionally, the success rate of the task could possibly be improved by training younger mice without head-fixation. As such, an alternative training method could be to use a mouse harnesses to position the young mice in front of the lick ports. This could help the mice have ample time to learn the contingencies related to the task prior to receiving head bar surgery.

Another method that our lab is in the initial stages of developing is a home cage behavior training system where mice can train at any time of day. Even though our current 4-box training setup is used to train 4 mice simultaneously, this training scheme is

low-throughput and labor intensive, with the timing of experiments limited by the animal trainers' schedules. In the future, we aim to implement an automated high-throughput training system. This set-up, which will be available to mice 24/7, will offer not only a greater number of mice access to the training task, but also gives mice flexibility in terms of desired task training duration.

Future Directions

We believe that devising a pattern separation task that initially presents simultaneous tones in the form of a chord (i.e. a template chord and non-template chords) in lieu of a sequence of tones (i.e. a template song and non-template songs) may present two notable advantages. First, mice would not be tasked with having to pay attention throughout the entire duration of each song to decide whether the presented sequence was 'template' or 'non-template'. Instead, mice will be able to respond immediately following the presentation of a simultaneous chord. These simultaneously-presented tones (chords) could then be increasingly staggered until they are presented as a temporal sequence of tones (a song). Secondly, we believe that mice may need a two-week period of time to grow accustomed to the task itself, so a chord-based training system may allow mice to gradually ease into a fully-realized pattern separation task that tests the temporal aspects of pattern separation. For example, each mouse would first be trained to discriminate between template and non-template chords. Then following mastery of this concept, each mouse will have the necessary understanding of the task to learn songs.

Before we are able to explore cord presentation, further improvements to our custom electronics hardware need to be made. Simultaneous tone presentation, though

something that has piqued our interest, has eluded us due to the technical complexity of presenting two or more pure tones simultaneously. Our lab is currently working on improving and upgrading our electronics setup to explore this variation of the temporal pattern separation task.

Additionally, one of our long-term goals is to perform two-photon imaging in two different regions of the mouse brain simultaneously. Many studies of pattern separation focus on the contribution of the DG. However, it is possible that other higher cortical areas could also be involved in pattern separation. For example, imaging the DG and its output region, CA3, could shed light on the true pattern separation ability of the DG. Thus, a more complete understanding of pattern separation will likely require the use of two-photon microscopy to simultaneously image the DG and cortex. Although imaging at two different depths simultaneously in a mouse brain using two-photon microscopy is not currently possible, our lab is currently working to developing this technology.

Understanding the mechanisms underlying hippocampal function, such as pattern separation, will be critical for our understanding of questions of memory such as why our ability to remember declines as we age. As technological advancements allow us to explore more complex questions, we can look forward to these insights having broader implications for memory impairment disorders, such as Alzheimer's and Huntington's disease, that are not well understood.

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