EXAMINING THE EFFECTS OF DEEP BRAIN STIMULATION AND TARGETED MEMORY REACTIVATION ON RODENT MOTOR LEARNING

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Abstract

One important form of memory that is enhanced through repeated training and sleep are motor memories. Motor memories play a vital role in many physical movements and skills we make. Despite this, our understanding of how these memories are processed and enhanced during sleep is not completely understood. Two techniques for sleep manipulation have been proposed to possibly help improve learning: deep brain stimulation (DBS) and targeted memory reactivation (TMR). This work tested whether a combination of these two techniques could improve the rate of learning in a pellet reaching task using rats. Four experimental rats who received DBS and TMR and two control rats were implanted with local field potential (LFP) electrodes and trained on the single-pellet reaching task for 15 days total, accompanied by rest before and after. Although DBS did increase the rate of spindles occurring in experimental rats, there was no performance difference in the task between groups. Sharp wave-ripple events did not significantly differ between groups, but spindle onset relative to DBS pulse peri-event histogram showed a significant increase at the 100-300ms range, supporting a previous study (Eckert et al., 2021). Follow-up pilot studies were conducted to further improve the original experiment's design as well, but no follow-up study design could be confirmed yet. Future directions are discussed including possible changes to stimulation paradigms, experiment design and using an alternative motor learning task.

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List of Abbreviations

DBS – Deep Brain Stimulation

EEG – Electroencephalogram

FBN – Fischer-Brown Norway

LFP – Local Field Potential

NREM – Non-rapid Eye Movement

REM – Rapid Eye Movement

SWR – Sharp Wave-Ripple

SWS – Slow Wave Sleep

TMR – Targeted Memory Reactivation

QW – Quiet Wakefulness

1. Introduction

1.1 General Theories of Sleep

Sleep is a state of the brain and mind that is generally characterized by a loss of consciousness, physical immobility and novel brain processes versus waking. Sleep is homeostatically regulated, such that deprivation of sleep is typically followed by prolonged sleep. Sleep-like behaviour is present in many species (Cirelli & Tononi, 2008); however, its function and mechanisms are not fully understood. A few theories with empirical support have been proposed for different purposes of sleep.

Firstly, sleep may act as an alternative state to wake for restorative processes (Eugene & Masiak, 2015). For example, Xie et al. (2013) found that β-amyloid, a form of metabolic waste, was cleared out faster during periods of sleep than during waking periods via the glymphatic system. This restorative process may have protective effects against neurological diseases such as Alzheimer's disease, however the relationship of β-amyloid clearance and Alzheimer's disease is ambivalent (Long & Holtzman, 2019). Sleep is also associated with periods of decreased body temperature and a lowered metabolic rate (Berger & Phillips, 1995).

Secondly, sleep may play a role in memory processing and learning (Diekelmann & Born, 2010; Frankland & Bontempi, 2005; Marr, 1970). Versus a period of wakefulness, sleep has been correlated with increased performance on declarative and procedural tasks, increased retention of explicit information and improved emotional memory (Diekelmann & Born, 2010; Tononi & Cirelli, 2006). More details on sleep and

memory are discussed in section 1.4 Memory Consolidation, and this purpose of sleep will be focussed on in this thesis.

Despite not understanding fully all the purposes of sleep, it is clear that it is a fundamental behaviour of many organisms considering its ubiquitous status (Cirelli & Tononi, 2008). Its presence and shared properties across species through natural selection, such as the presence of similar sleep stages in humans and birds via convergent evolution, has also been studied (Roth et al., 2010), noting its importance.

1.2 Sleep Stages

Sleep in humans and rodents is not a homogenous state, but rather is composed of multiple stages (Aserinsky & Kleitman, 1953; Diekelmann & Born, 2010; Loomis et al., 1937). Broadly there are two main phases of sleep, rapid eye movement (REM) sleep and non-rapid eye movement sleep (NREM). Typically, during a period of sleep the occurrence of NREM sleep and REM will occur in successive cycles (Rechtschaffen, 1968). Early portions of sleep have relatively longer NREM periods and shorter REM sleep periods (Aserinsky & Kleitman, 1953). Conversely, later portions of sleep have relatively longer REM periods, and shorter NREM periods.

NREM sleep is defined as a phase of sleep characterized by a high amplitude of slow oscillations in the cortex (Achermann & Borbély, 1997) (more on slow oscillations in section 1.3 Electrophysiological Properties of Sleep). There is a lower level of firing across the neuraxis of the central nervous system and PET scans also show a decreased amount of cerebral energy metabolism during NREM (Hobson & Pace-Schott, 2002). Specifically the sub-phase of NREM known as N3 can be considered a “deep” stage of sleep, and is comparable to a high dose of anaesthesia, exhibiting prolonged periods of

cortical down-states for example (Alkire et al., 2008). In this thesis work, I will be referring to rodent SWS which is analogous to human NREM.

REM sleep is defined as a phase of sleep characterized by a loss of muscle tone and wake-like cortical activity despite being in an unconscious state, hence also being known as “paradoxical sleep”. As opposed to NREM sleep, during REM sleep the hippocampus has relatively weaker low frequency power in the delta/slow oscillation bands and higher power in the theta frequency band. Being similar to waking activity, the cortex in REM sleep is described as being in a “desynchronized” state where there is lower coordinated population activity (Harris & Thiele, 2011).

1.3 Electrophysiological Events of Sleep

In addition to the electrophysiological properties of sleep described in section 1.2 Sleep Stages, there are a few important electrophysiological events putatively related to memory processes and detectable using local field potential recording techniques (which will be employed in this experiment, see section 2.2 Surgery for more information).

1.3.1 Spindles

Spindles are a cortical EEG event described by a brief 10-15 Hz oscillation in humans (De Gennaro & Ferrara, 2003; Loomis et al., 1935). Spindles originate from the thalamus due to an interaction of GABAergic neurons and glutamatergic thalamo-cortical projections (Diekelmann & Born, 2010; Timofeev & Bazhenov, 2005). Spindles typically occur during NREM sleep, but if they occur during SWS specifically, then they are typically seen around a cortical up-state transition of a slow oscillation. Conversely, in SWS during cortical down-states there is a suppression of spindle events, therefore slow oscillations play an important role in entraining the timing and occurrence of spindle

events (Hobson & Pace-Schott, 2002; Mölle & Born, 2011; Mölle et al., 2009). The transition from a cortical down-state of hyperpolarization to an up-state of depolarization is predicted to act as a synchronizing signal for the initiation of spindles. This depolarizing burst of neuronal activity and subsequent spindle is known as a K-complex (Hobson & Pace-Schott, 2002), and becomes relevant in section 1.7 DBS.

A critical property of spindles is the increase of their occurrence in sleep following learning (Briere et al., 2000; Morin et al., 2008; Rasch & Born, 2013), implicating an important role in sleep-dependent memory consolidation (see section 1.4 Memory Consolidation for more). In Mölle et al. (2009), they found an increase in spindles after learning occurred mostly during the cortical up-state in humans, but not necessarily for rats. For an example of what kind of increase is seen, Eschenko et al. (2006) found that during post-learning sleep in rats, there was an approximate 25% increase in spindle density, and this effect was most prominent in the first 60 minutes of sleep. This increase in spindle density and time window for the effect is similar to human results which show an approximate 33.5% increase, with a window of the first 90 minutes of sleep (Gais et al., 2002).

1.3.2 Sharp Wave-Ripples

Sharp wave-ripples are electrophysiological events that are composed of two parts: a sharp wave, and a ripple. Sharp waves are fast, depolarizing waves generated in the CA3 region of the hippocampus (Buzsaki, 2006). Ripples are 100-300 Hz oscillations that originate from the CA1 region of the hippocampus (Buzsaki, 2006). When a ripple is superimposed onto a sharp wave, then it becomes a sharp wave-ripple (SWR) event (Buzsaki, 2006; Rasch & Born, 2013). SWRs normally occur during SWS and periods of

no exploration such as quiet wakefulness. Similar to cortical spindles, SWRs typically occur during cortical up-states, and are suppressed during cortical down-states (Mölle et al., 2009).

A key property of SWRs is their correlation with reactivation of cell assemblies corresponding to previous waking experiences (see section 1.4 Memory Consolidation for more), suggesting a role of SWRs in sleep-dependent memory consolidation. For example, a human study has shown that the number of ripples during post-task sleep correlated with memory of pictures learned (Axmacher et al., 2008). Specifically, SWRs are believed to be critically involved in the consolidation of hippocampus-dependent memories, such as for spatial tasks (Girardeau et al., 2009; Rasch & Born, 2013; Wilson & McNaughton, 1994).

1.4 Memory Consolidation

Continuing from the memory processing and learning theory of sleep in the first section, I will discuss this in further detail while referencing the discussed sleep stages and electrophysiological events in sections 1.2 and 1.3.

It is thought that the different sleep stages have separate and synergistic effects on different aspects of memory processing. According to the “dual-process hypothesis” (Maquet, 2001), REM and SWS play separate roles in processing certain types of memories. For example, REM sleep deprivation by itself has mixed research on its direct effects on memory processing (Diekelmann & Born, 2010), however, varying the length of REM sleep has demonstrated its benefit to procedural memories and emotional aspects of memories though (Wagner et al., 2002; Wagner et al., 2001). On the other end, varying levels of SWS has demonstrated an effect on enhancing specifically declarative

memories. Some research has also suggested that both REM and SWS aid with memory processing of each other's respective memory types, providing evidence for the importance of alternating sleep phases in general, also known as the "sequential hypothesis" (Giuditta et al., 1995).

Key to sleep in general though is the mechanism of memory reactivation, and its critical role in sleep-dependent memory consolidation (Diekelmann & Born, 2010; Frankland & Bontempi, 2005; McClelland et al., 1995; Wilson & McNaughton, 1994). Firstly, memory consolidation is the process of converting a short-term, labile memory from an experience into a long-term, stable memory trace. Memory consolidation is a process, and its core mechanism of operation is through memory reactivation during periods such as sleep. Memory reactivation is the process of the neural circuits involved in the original encoding of a memory or experience becoming re-activated during offline periods (McClelland et al., 1995). Memory reactivation normally occurs during SWS in a manner that is sped up versus the original experience (Skaggs et al., 1996), with a fraction of the original neurons involved, and mostly within the first hour of sleep after learning (Kudrimoti et al., 1999). Through a process called "active system consolidation", it is theorized that memory traces are re-activated in the hippocampus (McClelland et al., 1995; McNaughton & Morris, 1987), which drives a similar re-activation in the neo-cortex to consolidate the memory into a long-term format (Skaggs & McNaughton, 1996; Wilson & McNaughton, 1994).

Regardless of the exact mechanism by which consolidation occurs, it is strongly supported that the presence of reactivation in SWS plays a causal role in memory consolidation (Diekelmann & Born, 2010; Johnson et al., 2010; Rasch et al., 2007). The

modulation of this memory re-activation may play a role behind the methods and predictions of this thesis experiment.

1.5 Motor Memory

Transitioning from memory consolidation in general to a specific form of memory, I will discuss procedural memory and then specifically motor memory. Procedural memory is a type of nondeclarative memory that pertains to skills that are implicitly learnt through practise, and typically requires multiple repeated training sessions to learn (Rasch & Born, 2013; Squire & Zola, 1996). The nondeclarative nature of procedural memories entails that these types of memories are not always consciously retrievable. Examples of tasks that utilize procedural memory are the perceptual discrimination task (Robertson et al., 2004) and parts of language learning, (for example, Peigneux et al. (2001) suggests understanding correct grammar without an explicit understanding of the rules is implicit learning of a procedural memory).

One common type of procedural memory studied is motor memory. Motor memories entail the memory of skilled movement of the limbs and/or body for some goal directed behaviour. For example, riding a bicycle and playing a musical instrument require motor memory of gross and fine movements, respectively (King et al., 2017). For this thesis experiment, the rodent single-pellet reaching task (Whishaw & Pellis, 1990) was utilized, which is the task of learning to perform a fine movement of the arm and paw to reach through a slit and grab a sugar pellet, then bringing it back to their mouth (methodology described in detail in section 2.4 Single-Pellet Reaching Task).

The mechanisms by which motor memories are processed and consolidated in humans is still not fully understand due to conflicting results and nuanced differences in

motor tasks utilized (King et al., 2017). However, for the single-pellet reaching task in rodents, it has been confirmed that post-learning sleep plays a role in learning and is modulated by performance during the task (Eckert et al., 2020; Hanlon et al., 2009; Ramanathan et al., 2015). However, it is unclear whether there is hippocampal involvement in the reaching task. Involvement of the hippocampus in human motor tasks is dependent on the type of task, where evidence has been found for a role of the hippocampus in learning of motor sequence tasks (e.g. serial reaction time task), but not for motor adaptation (e.g. mirror tracing) or continuous tracking tasks (e.g. pursuit rotor task) (King et al., 2017).

1.6 TMR

Transitioning from an overview of sleep and memory in their natural state, I want to introduce two techniques that have been used in attempts to modulate learning and sleep. I will start with Targeted Memory Reactivation (TMR), and then in the next section discuss Deep Brain Stimulation (DBS).

TMR is the technique of pairing a cue with a learning experience, and then re-exposing the user to the cue during sleep. The cue can be an odour or a specific sound for example, which is administered when a participant is training on a learning task. A paired association is created through this exposure, which then is predicted to affect memory consolidation of that experience if presented during sleep. An early and influential study on TMR was performed by Rasch et al. (2007). At this time, it was known that odours were a relatively effective cue for memory retrieval (Chu & Downes, 2002), so they paired a visuospatial task with an odour cue during training, demonstrating improved performance after odor delivery during sleep versus a group that did not receive the

paired odor during sleep. An important aspect of this finding was that this effect was demonstrated for a declarative task that also involved the hippocampus. Later research showed that TMR putatively increases reactivation of the associated learning experience but does not increase memory reactivation in general during sleep (Rudoy et al., 2009). Therefore, TMR can be described as a potential method of biasing memory replay during sleep towards a specific experience, and through increased consolidation, potentially improving the rate of learning.

A critical paper studying TMR in rodents that part of this thesis work is based on was performed by Bendor and Wilson (2012). This study trained rats on an auditory spatial task to move left or right after a nose-poke while one of two cues was played. During post-task sleep, both sound cues were intermittently replayed, and it was demonstrated with unit activity that replay of the left and right movements were increased during NREM sleep. However, replay did not necessarily occur when the audio cues were delivered. Therefore, it was suggested that TMR increased the likelihood of replay occurring during a short window (in this experiment's case, about 10 seconds).

Further research has been conducted with TMR, finding performance generally increased when TMR was delivered during SWS, but not during REM sleep (Hu et al., 2020). For example, Laventure et al. (2016) found that delivering a task-associated odour during human stage 2 sleep increased performance on a procedural finger-sequence task, but not for participants who received the odour during REM sleep. Hu et al. (2020) also found in their meta-analysis of human TMR studies that TMR can be effective in both declarative memory and skill acquisition tasks.

For more examples of recent human TMR research, Larry et al. (2021) demonstrated improved performance on a muscle feedback task which was considered a type of novel skill learning independent of the hippocampus. An interesting aspect of this study was that the later phases of training removed any visual components of the task and instead participants only had the sound cue to instruct specific actions, thus strengthening the association between specific sounds and aspects of the task for later TMR delivery. Works such as Boutin and Doyon (2020) and Antony et al. (2018) also propose the importance of timing of TMR delivery during SWS. Boutin and Doyon propose a "mesoscale" oscillation of 0.2-0.3Hz that determines the timing of spindle events within observed groups of spindles known as "spindle trains", where these spindle trains have shown some implications for motor learning. Antony and colleagues (2018) considered the period between these spindles in spindle trains a "refractory period" and found that delivery of TMR outside of these refractory periods was effective at improving a spatial memory versus delivery during them. Therefore, it appears that some recent human TMR research is attempting to investigate the nuances and methods behind more effective TMR in learning.

However, the effect of TMR on motor skill learning in rodents is less clear. There have been no direct replications of the Bendor and Wilson (2012) study to substantiate the link of TMR and altered behaviour. However, there was work performed by Rothschild et al. (2017) that supports the mechanism of TMR inducing a change in neural activity during sleep. They tested whether TMR via a tone associated with a task could bias the activity of the auditory cortex, which in turns would affect the activity of the hippocampal CA1 region. It was confirmed that the TMR tone did successfully bias the

auditory cortex (versus control tones) with cell ensemble reactivation of the task and was associated with later CA1 reactivations. The authors proposed that this may be the mechanism behind how Bendor and Wilson (2012) observed a biasing of hippocampal reactivation from auditory stimuli. Nonetheless, Rothschild et al. (2017) did not compare behavioural or learning changes in rats due to the TMR tone, so the question of whether TMR can affect procedural motor learning in rats is unanswered.

1.7 DBS

Before considering DBS, I would like to cover electrical stimulation and Transcranial Electrical Stimulation (TES) and its related variants. Considering neural communication and action potentials are in part electrical phenomenon, it is possible to modulate the behaviour of neural systems with an external application of electrical stimulation, which can modify firing rates due to altered neuron membrane potentials for example (Vöröslakos et al., 2018). The electrical stimulation can come in different forms such as through skull surface electrodes or via implanted electrodes and can be delivered as alternating current or pulses of direct current for example.

An early study that implicated a possible application of electrical stimulation to memory processing and learning was performed by Marshall et al. (2006). Marshall and colleagues tested whether transcranial electrical stimulation delivering a 0.75 Hz slow oscillating potential during early sleep could affect performance on a hippocampus-dependent paired association task. They observed an increase in SWS and improved performance on the memory task versus a control group with sham stimulation. An important property of their protocol was that stimulation was capped at approximately 0.52 mA.

However, the mechanism and validity of TES in work such as Marshall et al. (2006) was challenged with the publication of work examining the stimulation and response of neural structures in human cadavers by Vöröslakos et al. (2018). This study determined that a minimum of 4mA is required to induce change in underlying neural activity when delivering TES via electrodes on the scalp, and that most of the current is dissipated by the skin and other intermediate tissue. The threshold that Vöröslakos and collegues determined is above the typical strength of TES in studies and application, including the protocol used by Marshall et al. (2006). Vöröslakos and collegues proposed that perhaps previous work that claimed neural activity was altered by TES failed to account for large stimulation artifacts, among other reasons.

However, Vöröslakos et al. (2018) does not explain the mechanism behind positive results of TES found on memory tasks across multiple studies. Barham et al. (2016) performed a meta-analysis on 13 human TES studies and concluded that TES can be effective at enhancing or disrupting declarative memory tasks, but found no effect for procedural memory tasks.

An interesting connection between TMR and TES studies however has been the putative success of inducing memory changes when delivering during SWS as opposed to REM sleep (Barham et al., 2016). Majority of TES studies have focussed on delivery during SWS, but studies such as Marshall et al. (2011) and Nitsche et al. (2010) looked at whether TES during REM could be effective for improving procedural memory consolidation. Only Nitsche et al. (2010) found an effect, but the caveat is that this effect only appeared if particpants were woken immediately after TES delivery and not after

completing regular sleep overnight. Nonetheless, the putative successes of TES and TMR may both lie in their delivery during SWS.

However, considering the claim from Vöröslakos and collegues challenging the behavioural successes of Barham et al. (2016)'s review, there continues to be debate and need for further research about the effectiveness of TES on inducing changes in neural activity or behaviour, especially regarding procedural tasks.

DBS on the otherhand, is an alternative to TES in how it aims to deliver electrical stimulation via implanted electrodes, therefore making direct contact with targeted brain regions. DBS has found succesful application in clinincal settings, such as helping alleviate symptoms of Parkinson's disease (Benabid, 2003; Deuschl et al., 2006), drug-resistent epilepsy (Li & Cook, 2018) and severe depression (Wichmann & DeLong, 2006). The mechanism by which DBS operates is not fully understood, but it is predicted by some researchers that delivery of a DBS signal helps replace intrinsic neural signals, and thus disrupting flow of pathologic bursting and oscillations, reducing disease symptoms (Chiken & Nambu, 2016; Johnson et al., 2008). DBS has also been employed in rodents, testing its impacts on learning and memory. For example, Vyazovskiy et al. (2009) found that DBS delivered during early sleep via brief electrical pulses in rats induced slow wave oscillations.

A key study for this thesis work utilizing DBS was performed by Eckert et al. (2021). Using four Fisher-Brown Norway (FBN) rats with an implanted electrode in the motor cortex, stimulation was delivered during SWS during a stimulation epoch. LFP activity was recorded during this stimulation epoch as well as non-stimulation rest periods before and afterwards for comparing baseline and resulting activity, respectively.

Stimulation itself consisted of a 300µs biphasic pulse at 500µA, delivered every 3 seconds during confirmed real-time SWS. The resulting LFP behaviour exhibited an increase in both slow oscillations by 30% and spindle events by 37% versus periods with no stimulation delivered. The effect of increased slow oscillation and spindle events did not continue once stimulation was stopped though. Also importantly, it was verified that stimulation did not affect the amount of sleep that occurred.

Even though Eckert et al. (2021) compared and found evidence that the waveform of the evoked spindles were largely comparable to endogenous spindles, it has not been confirmed if these evoked spindles exhibit known spatiotemporal propatation through the cortex. Piantoni et al. (2017) observed in a human experiment a trend of spindles generally propagating from the superior frontal region (including the sensory and motor cortices) towards the orbitofrontal cortex. Although in vitro brain slice work found evidence that electrically induced spindles propagate faster than endogenous spindles (Kim et al., 1995), it is not obvious in the current literature whether DBS evoked spindles in vivo have the same spatiotemporal propagating behaviour as endogenous spindles. Also, with the LFP surgery setup in Eckert et al. (2021) and in this current thesis work, it cannot be confirmed whether this propagating behaviour is also observed for the evoked spindles due to a lack of fine spatial recording sites, especially in the frontal region. Nonetheless, the DBS paradigm proposed by Eckert et al. (2021) may still behave like endogenous spindles and thus potentially be involved in memory consolidation processes.

The protocol developed by Eckert et al. (2021) for DBS stimulation is the basis of the method used in this thesis work. Considering the mixed results and challenges of TES/DBS studies, a critical next step and question (which will be expanded on in the

following sections) is whether this increase in slow oscillations and spindles can affect learning and behaviour in rats.

1.8 Past Studies in the Tatsuno Laboratory

Before explaining the formal purpose and theory of the current thesis experiment, I want to give some context from previous studies conducted in the Tatsuno laboratory leading up to this project. In order to investigate the viability of TMR on improving motor memory and learning in rodents, two previous unpublished studies were conducted in the Tatsuno laboratory in 2015 and 2017 utilizing TMR and the single-pellet reaching task.

In the 2015 study, 12 FBN rats were split evenly into a control group and an experimental group. Both groups performed the single-pellet reaching task everyday for 14 consecutive days, with up to 20 reach attempts (or trials) per day. During reach training, whenever a reach attempt was made, a tone was played to create an association with the act of reaching. After reach training, rats were subjected to post-training rest in a nearby cage. The experimental group also received intermittent tone playback during this rest period for the purpose of TMR. The reaching success of this experiment is shown in Figure 1. No statistical difference was found in reaching success between the control and experimental group.

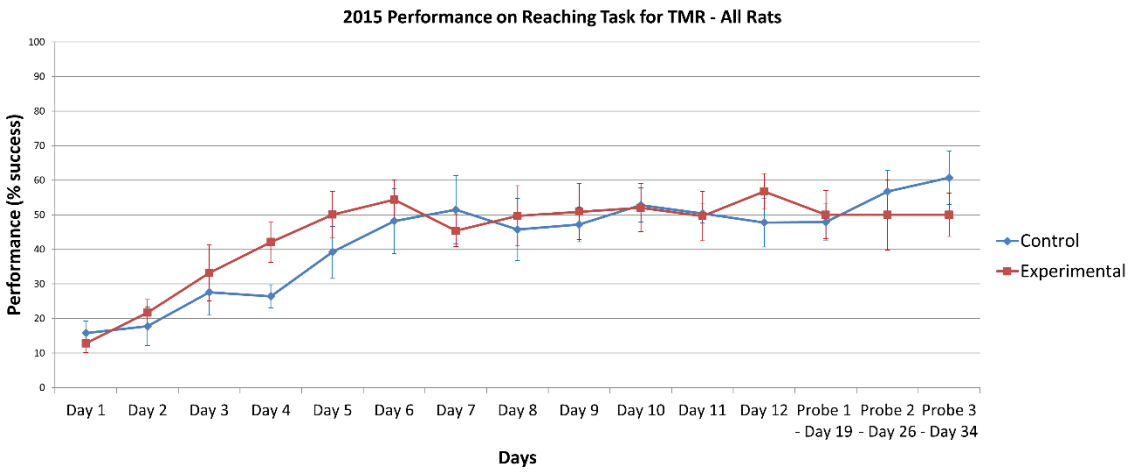

Figure 1. Reaching performance for 2015 study. Error bars represent SEM.

However, there is small visual evidence that learning was faster in the experimental group during the 3rd to 6th days of training. This led to a prediction that perhaps TMR is able to increase the rate of learning, but not necessarily increase the maximum ability after training hits a plateau. This experiment also did not utilize electrophysiology recording, and therefore was not able to verify whether the rats were asleep during TMR delivery, let alone the sleep stage and presence of spindles/SWRs.

The 2017 study was an attempt to build on the 2015 work with a fleshed-out design. A very similar design of 12 FBN rats split into a control and experimental group was used, but LFP recordings were incorporated. LFP recordings were made from the hippocampi and primary motor cortex. Single-pellet reaching was conducted over 12 days and included a probe trial a week after the last day of regular training ended. All rats again received a tone during reaching, and the experimental group received these tones during post-task rest. However, using the LFP recording, a real-time estimate of sleep stage was used to only deliver the TMR tones during SWS. Reaching success results are

shown in Figure 2. Again, no statistical difference was found in reaching success between the control and experimental group.

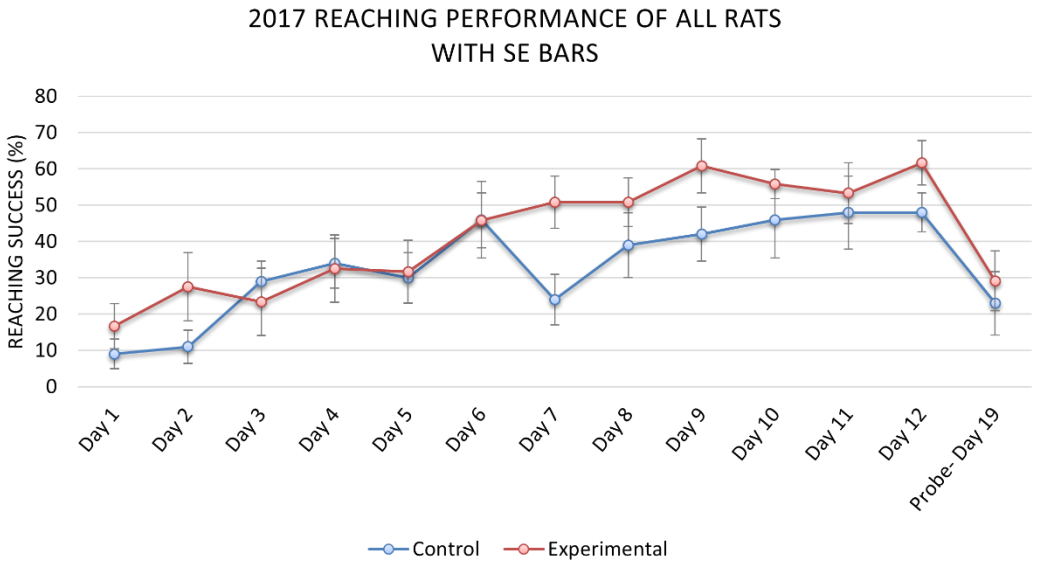

Figure 2. Reaching performance for 2017 study. Error bars represent SEM.

Unfortunately, there is no evidence of a similar bump in performance around the 3rd to 6th day period like in the 2015 study. Despite the added precision of delivering TMR during confirmed SWS periods, there does not appear to be an effect of TMR on the rate of learning or peak performance level. However, this experiment suffered from a critical issue of relying on multiple experimenters conducting the training and sleep recording of the rats. It is therefore possible that different experimenters handled, delivered pellets during reach training, and classified success/fails differently from each other. There was also the realization that the delivery of the TMR tone during reach training may have been problematic. The TMR tones were played automatically whenever a rat's paw crossed an infrared beam sensor placed in front of the pellet. This

means that the tone would always play during the reach, and thus potentially after preparations and decisions were made about reaching for the pellet.

1.9 Experiment Overview

In an effort to build on the 2015 and 2017 TMR studies conducted in the Tatsuno laboratory, the current thesis project was conceptualized. TMR has showed some promise in enhancing memory related to procedural tasks in humans, but there has been a lack of work attempting to replicate the behavioural findings of Bendor and Wilson (2012) in rodents. There is also a lack of understanding in whether the procedural memory enhancement results in humans can extend to specifically motor memory tasks in rats as well. Regarding DBS, the question remains whether the protocol introduced by Eckert et al. (2021) can enhance behavioural performance on a learning task. Therefore, TMR and DBS exist as two potential techniques of modulating sleep to enhance learning, specifically for a procedural motor task. Testing whether these techniques can affect rodent behaviour and electrophysiology would have implications in better understanding their efficacy, mechanism of action, and perhaps inform future rehabilitation research in improving the motor learning process of stroke victims for example.

This brings up the theory, hypothesis and research question for this experiment. The main theory this work relies on is that waking experiences and skills are processed from a labile state to a long-term stabile state during sleep via memory consolidation, which is driven by the process of memory reactivation. My hypothesis is that this process of memory consolidation during sleep can be enhanced by a combination of TMR and DBS delivery for a procedural motor memory task. More specifically, I hypothesize that increasing the rate of sleep spindles via DBS, and biasing memory replay through TMR

will increase offline gains for motor learning. To address this hypothesis, my research question was: will rats that receive both DBS and TMR during sleep exhibit faster learning and/or higher overall performance on the reaching task than a control group?

2. Methods

2.1 Subjects

The original design was to utilize 24 male Fischer-Brown Norway hybrid rats. However, only 6 rats were tested (reasoning explained in section 4.1 Experiment Redesign Justification). The FBN breed was chosen based on their tendency to sleep more than most other breeds, which would make sleep recordings and manipulation more effective during testing. The FBN rats were obtained from both the University of Lethbridge breeding colony and ordered from Harlan Inc. (IN, USA). Of the 6 rats tested, 5 were 17 months old and one was 20 months old. Weight information will be provided in next version of thesis once available. The terminology “all rats” will now refer to the 6 tested rats from here forth. All rats were housed using a 12:12 hour dark/light cycle, and all testing and sleep recordings occurred during the light portion. On the first day of the experiment, all rats began food restriction, targeting a body weight between 85-90% of their starting body weight. There was ad libitum access to water throughout the experiment. Work was conducted under protocols 1709 and 2110, which were approved by the University of Lethbridge Animal Care Committee and followed the guidelines stated by the Canadian Council for Animal Care. Please refer to the Appendix for an explanation about the lack of female rats and combination of TMR and DBS into one group.

2.2 Surgery

The 6 rats underwent electrode implantation surgery while anesthetized with 2-3% isoflurane. Four bipolar local field potential (LFP) electrodes were implanted along with one ground screw. The cortex recording electrode was specifically targeting region

M1, cortex stimulation electrode was implanted into the corpus callosum (inferior to M2), and both hippocampal electrodes were specifically targeting CA1. Further details of the electrode's region, coordinates, and bipolar tip separation are in Table 1. An illustration of the electrode implant locations is also shown in Figure 3. Using the paw assessment results, the motor and stimulation electrodes were always implanted contralaterally to the dominant paw, so that the hemisphere largely responsible for reaching motion was being recorded and stimulated. After surgery, all rats were allowed to recover for at least a week before testing resumed.

Electrode Name	Coordinates	Tip Separation
Left Hippocampal LFP	3.5 mm posterior, -2.5 mm lateral, 2 mm deep	0.5 mm
Right Hippocampal LFP	3.5 mm posterior, 2.5 mm lateral, 2 mm deep	0.5 mm
Motor Cortex LFP	1 mm anterior, ±3 mm lateral, 2.6 mm deep	1.5 mm
Motor Cortex Stimulation	1 mm anterior, ±1.8 mm lateral, 2.5 mm deep	0.5 mm

Table 1. Details of the four electrodes implanted per rat. Coordinate positions are all relative to bregma. Motor Cortex LFP and Motor Cortex Stimulation electrodes were always implanted on the same side together while also being contralateral to the dominant paw.

One new component of this experiment versus previous work in the Tatsuno laboratory was the use of an accelerometer to measure motion. Instead of using a neck

muscle EMG electrode, an accelerometer built into the head stage was utilized. The accelerometer records changes in acceleration in 3 dimensions, and this data was recorded as a 2000Hz signal by the Cheetah recording system (see Section 2.6 Hardware). The accelerometer has a notable advantage over a neck EMG. Neck EMGs are subject to greater variation and noise in the signal due to subtle differences in implant location and the nature of biological recordings. In contrast, the same accelerometer was used for all rats, ensuring that the signal for estimating motion was consistent and comparable for all rats. Not requiring a neck EMG also simplifies surgery and frees up room for recording from more LFP channels elsewhere.

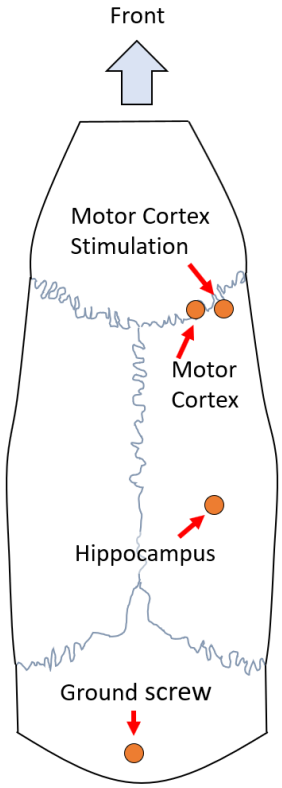

Figure 3. Diagram of LFP electrode locations on a rat skull. Image is viewed from a top-down perspective of the skull surface. Skull sutures are shown in light blue traces.

2.3 Schedule Design

The experiment was conducted on cohorts of 6 rats at a time. An overview of the schedule per cohort is detailed in Table 2. Also note that throughout all phases of the experiment, each rat was tested in the same order and at approximately the same time each day.

Phase	Time
Pre-training Habituation	5 days
Paw Assessment	2 days
Surgery	18 days
Baseline Recording	3 days
Training	14 days
Probe Trial	7 days after last day of training

Table 2. Chronological summary of experiment design.

Pre-training Habituation Phase entailed habituating the rats to the novel experimental room and cages. Each day for 5 days, each rat was exposed to the reaching cage for 15 minutes and the sleeping cage for 45 minutes. During reaching cage habituation, 7 sugar pellets to be used in the single-pellet reaching task were placed around the floor to be habituated to as well.

Paw Assessment Phase entailed testing each rat to determine which paw was dominant and preferred for the reaching task. Rats were naïve to the single-pellet

reaching task, so pre-training experience was minimized by limiting the number of attempts up to 10 a day. Each of the potential 10 attempts was not a full single-reach, but instead simulated the start of a reach by having the experimenter lure the rat to the reaching area and raise one of its paws towards a pellet held in front of their nose. Each paw that was raised was recorded, and if one paw was raised at least 70% percent of the time, then this paw was deemed the dominant paw. If there was no consistent paw for the first day, a second day of paw assessment was conducted under the same conditions.

Surgery Phase entailed implanting LFP electrodes into all rats for the cohort over an approximate 18-day period (including recovery time). This phase also included LFP test recordings to ensure adequate signal quality. Further details are outlined in section 2.2 Surgery.

Baseline Recording Phase entailed 3 days of daily LFP recording while the rats were in the experiment room. Specifically, each day the rats were first subjected to 15 minutes of recording in the reaching cage. Seven pellets were again placed on the floor of the cage. The experimenter also sat next to the cage during reaching cage baseline recording to simulate the later reaching task scenario. The purpose of this phase was to both obtain a baseline of recording data from waking and sleep sessions before experimental manipulation began, and to adjust the real-time SWS detection algorithm to be tuned to each rat.

Training Phase entailed 14 days of single-pellet reaching and sleep recordings, marking the start of video collection and reach success recording. Each day of training consisted of three epochs in the following order: pre-task rest, task, and post-task rest. Pre-task rest epoch consisted of a 45-minute recording in the sleep cage. Task epoch

consisted of up to 15 minutes of single-pellet reaching for sugar pellets in the reaching cage. More details about this task are in section 2.4 Single Pellet Reaching Task. Lastly, the post-task rest epoch consisted of a 75-minute recording in the sleeping cage. The experimental room was lit during the pre-task rest and post-task rest epochs but used red lighting during the task epoch. The reason for the red lighting during the task was to help mitigate stress in the rats by simulating a nocturnal environment to perform in. Regular lighting during the rest epochs was utilized to match their accustomed circadian rhythm, therefore promoting sleeping behaviour. Once all 14 days of training were complete, the rats did not perform any more single-pellet reaching or recording for 6 days, but food restriction was continued.

Probe Trial Phase concluded the experiment for each cohort. It entailed a single day of regular pre-task rest, task, and post-task rest epochs on the 7th day after the Training Phase ended. The purpose of this phase was to test the retention of the learned reaching skill after a period of no practise.

Regarding daily scheduling of events, each rat was tested at approximately the same time of day. Rats were also always tested in pairs using two recording rooms in parallel. However, the reaching task was always performed in the same room for every rat, but rest periods were performed in two rooms. Identical recording equipment and rest cages were utilized in both rest rooms to remove any confounding differences. All rats used both rest rooms as well, but in reverse order between pairs. For example, both control rats were a pair, and one rat would start in the 1st rest room while the other rat started in the 2nd rest room. Both rats would then get at least 45 minutes of rest recorded, and then after reach training, the rats would switch rooms for post-task rest. The design

of running rats in pairs required that both rats be either controls or experimental to simplify the recording procedures and is therefore the reason for only 2 control rats and 4 experimental instead of a balanced number. This paired design across two rest rooms was utilized so that the cohort size could be increased due to its time efficiency with overlapping recordings.

2.4 Single-Pellet Reaching Task

The behavioural task used to assess motor learning was the single-pellet reaching task. Its design and execution are largely based on Eckert et al. (2020). The reaching cage was a translucent polycarbonate box with an open top and a 1.5cm open slit at the front. Through this slit, rats could sniff and locate the pellet (45mg; Bio-Serve, NJ, USA). However, they could not reach the pellet with their mouth, but instead had to learn to coordinate the motion of lifting, reaching out and grasping a sugar pellet with their paw and digits. The sugar pellet was placed in one of two wells on a platform in front of the slit. Depending on the dominant paw of the rat, the pellet was placed in the contralateral well to encourage reaching on a slight diagonal angle as opposed to straight ahead. During the task epoch, rats would get up to 20 reach attempts each day. An attempt is when a single pellet was placed in a well, a door blocking the slit was then opened, and the rat either makes a reach attempt or is inactive/distracted for at least a minute. A trial ends with closing of the door. Timing and how the door operated, along with how reach times were measured are explained in section 2.6 Hardware. If a rat continued to be consistently distracted or inactive during reach trials, the task epoch was prematurely ended before completing 20 reach attempts.

An important modification made to this task is the addition of a small 2.5mm high barrier between the reaching slit and the pellet well (Image 3). Pilot testing before the study demonstrated that some rats developed a reaching strategy of loosely cupping the pellet with their paw, and then dragging it back to the reaching slit. To encourage proper reaching behaviour of grasping and lifting a pellet, a barrier made of a cotton tip applicator handle was added to prevent dragging. The rat was forced to grasp the pellet to a higher degree in order to retrieve the pellet due to the barrier.

Success scoring of each reach attempt was recorded as either ‘success’ or ‘fail’. A ‘success’ was defined as when the rat the was able to grab or move the pellet such that it reached or touched their mouth, excluding hits which caused the pellet to accidently launch into the reaching slit. All reaches that were not a ‘success’ were therefore a ‘fail’.

2.5 Groups

Rats were split into two groups: control and experimental. Of the 6 rats tested, 4 were in the experimental group, and two were in the control group. The reason for the group imbalance is due to a practical consideration of the testing schedule and setup. Rats in a cohort were always tested in pairs at the same time. To simplify recording setup, stimulation delivery, and to also minimize human error, pairs of rats were always members of the same group.

Experimental group underwent two experimental manipulations: DBS and TMR. Experimental rats would receive TMR during the task epoch and receive DBS and TMR during post-task rest. Neither manipulation was delivered during pre-task rest.

The control group did not receive DBS at any time. Control rats did not receive TMR during the task epoch, however they did receive TMR during the post-task rest. The reason TMR was delivered during the post-task rest was because it acts as a control for the potential effect of a tone played on sleep duration and sleep quality between groups. As a reminder, the TMR tone for the control group was not associated with reaching. The control group also did not receive any manipulation during pre-task rest. A summary of the manipulations and their timing are in Table 3.

	Experimental		Control	
Pre-task Rest	TMR: ✗	DBS: ✗	TMR: ✗	DBS: ✗
Task	TMR: ✓	DBS: ✗	TMR: ✗	DBS: ✗
Post-task Rest	TMR: ✓	DBS: ✓	TMR: ✓	DBS: ✗

Table 3. Group manipulation summary. Checkmarks signal manipulation was present, X's mark an absence.

2.5.1 DBS Paradigm

DBS was delivered according to the protocol developed by Eckert et al. (2021) to the motor cortex stimulation electrode. The electrical signal was generated by a GRASS S88 Stimulator (Natus Medical Incorporated, ON, Canada), where each pulse consisted of a single biphasic pulse at 500µA for 150µs. Voltage and polarity of the DBS stimulation was tuned to each rat during the baseline recording phase so that the

minimally required strength needed to reliably induce spindles was selected. Resulting voltages were either 3.5v or 4v. The stimulation isolation unit was also set so that it converted the set voltage strength to a constant current, so that regardless of resistance changes in the stimulation electrode over time, the delivered current would be the same. DBS pulses were only delivered during post-task rest for rats in the experimental group. During recording, a MATLAB script (Version 2022b; MathWorks, MA, USA) was predicting when SWS was present using a high delta over theta power ratio in the hippocampal LFP, and low accelerometer signal power (signifying motionlessness). Once SWS had been detected for at least 3s, DBS pulses would begin being delivered every 3-4 seconds as long as SWS continued. DBS pulses were delivered on a uniformly random 3-4 second interval to avoid entraining any regular rhythms.

2.5.2 TMR Paradigm

TMR protocol is based in part on the method used by Bendor and Wilson (2012). A tone was delivered both during the task epoch for experimental rats, and during post-task rest for both groups. The duration and timing of the TMR tones differed based on the epoch.

During the task epoch, a pure 6000hz, 1.5s, 80dB tone was utilized. At the start of a reaching trial and before the reaching door opened, the tone would play for 0.5s. Then, as the door opens, the tone would continue for another 1s. The reason for this timing with the door is so that the sound of the door motor is not the main association with the reaching task, but rather the tone that plays before the door opens, but also continues as the door opens, thus also attempting to mask the motor sound. Another important note is that this timing of the TMR tone playing before the door opens and the reach begins is a

critical improvement in design over previous single-pellet reaching and TMR experiments conducted in the Tatsuno lab. These former experiments would have the tone play when the infrared beam was broken (see section 2.6.1 Door and Tone System for more information), but that is during the reach and therefore it could be argued that the tone is not associated with the initiation and/or entirety of the reach when it only plays partway through the action. For example, Murakami et al. (2014) found that some M2 neural unit activity predicted movement initiation ranging from a few hundred milliseconds to beyond a second before movement started. Considering that one of M2's outputs is to M1, it may be advantageous to time the TMR tone so that it occurs before or in parallel with preparatory neural activity for movements such as reaching.

During the post-task rest epoch, a pure 6000hz, 0.5s, 60dB tone was utilized. The quieter volume was chosen as to minimize any sleep disturbances. The tone was delivered 200ms after each DBS pulse (to align with average spindle onset after DBS is delivered) for the experimental group. For the control group, once SWS was detected in the same fashion as the check for DBS, a TMR tone was delivered using the same properties and uniformly random interval of every 3-4 seconds. Although section 1.6 TMR covered work that suggested greater effectiveness of TMR if the timing of delivery was tuned to endogenous spindle trains (Antony et al., 2018) or cortical up-states (Ngo & Staresina, 2022), the primary purpose of the TMR paradigm was to be more closely based on that used by Bendor and Wilson (2012) in an effort to replicate its methodology. Considering DBS is also already being combined with TMR in this work, it would also help simplify the already intricate stimulation paradigm present by not needing to adjust

the timing of TMR to endogenous events such as spindle trains, but rather only to more macro-scale events such as a period of SWS.

2.6 Hardware

All portions of the hardware and analysis code developed solely by me are publicly available on Github (Campbell, 2023). Some proprietary scripts developed by the Tatsuno laboratory were modified by me and employed as well but are not publicly hosted. This proprietary work includes the sleep stage classification scripts developed by Michael Eckert and used in Eckert et al. (2020). All LFP recordings were done with a Cheetah system (Neuralynx Inc., MT, USA) and all channels were sampled at 8khz (except for accelerometer channels sampled at 2khz).

2.6.1 Door and Tone System

A hardware system consisting of Arduino Uno Microcontrollers (Arduino SRL, Monza, Italy), a motor, LEDs and speaker were incorporated to augment the traditional single-pellet reaching setup. The experimenter can exercise finer control over the timing and recording of data during the task with this system. Using a switch, a reach trial can be initiated. The switch will trigger the tone to play, and then 0.5s later pull the door open, allowing the rat to reach through the slit. The timing of the tone and door open are sent as events to the Cheetah recording system. To detect when a reach is made, an infrared beam sensor is placed in front of the pellet's position in a well, such that in order to grab the pellet, the infrared beam must be broken. Whenever the infrared beam is broken, a beam break event is sent to the Cheetah recording system.

At the same time as these different events are sent to the Cheetah recording system, certain LEDs in view of the high-speed camera will also light momentarily to

signal the event to the video cameras (see next section). These LEDs allow the synchronizing of Cheetah event timestamps with specific video frames, connecting behavioural event timestamps to video frame timestamps. Image 1 shows the reaching cage with hardware beside it. Image 2 shows the hardware system without the cage. Note that three Arduino systems were utilized because true multi-processing is not possible with a single device, thus accuracy of different events and timing could be disrupted if reliant on a single thread.

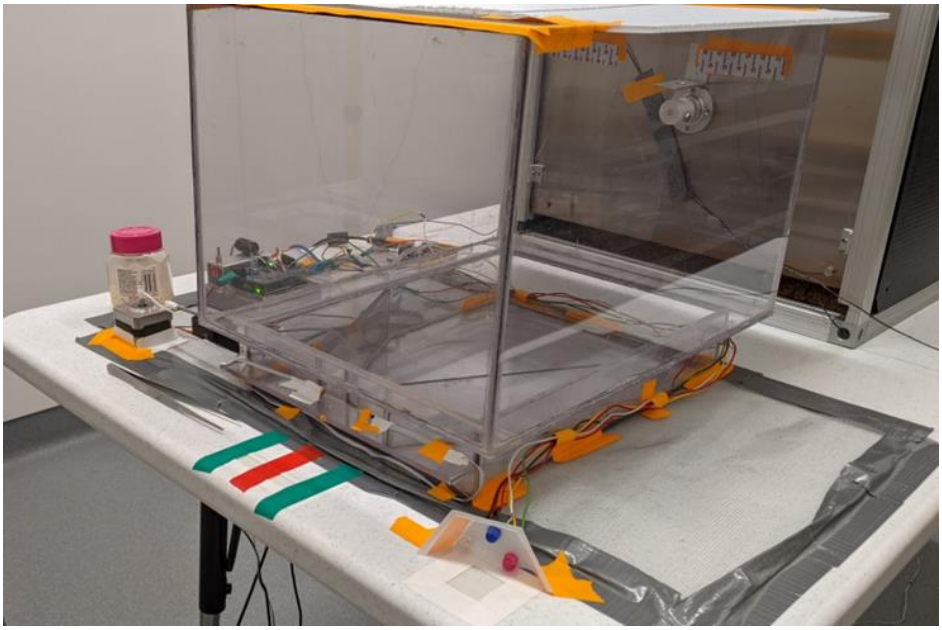

Image 1. Reaching cage. Majority of hardware is shown to the left of the clear cage. LED indicator system can be seen to the lower right of the cage, with three LEDs.

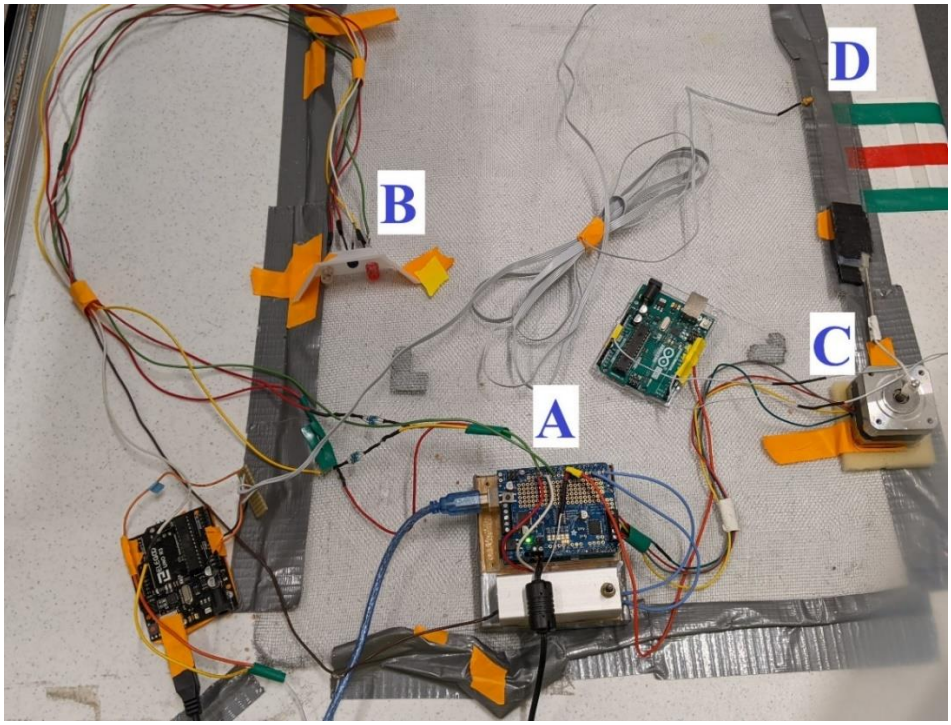

Image 2. Reaching hardware components. Arduino system (A), LED indicator (B), motor (C), and infrared beam sensor (D). Reaching cage was removed for photograph.

2.6.2 High-Speed Video Camera System

In addition to electrophysiology data and manual reach scoring data, the task epoch was also recorded with two synchronized, high-speed video cameras. The first video camera viewed the reaching from the front and acted as the main view of the reaching motion for later video analysis. The 2nd video camera had a side view that had the LEDs visible. Both video cameras were BLACKFLY S USB3 video cameras (Model: BFS-U3-16S2M-CS: 1.6 MP, 226 FPS, Sony IMX273, Mono) from Teledyne FLIR LLC (OR, USA), and recorded at 100fps, 1440x1080 pixels in monochrome. The video cameras recordings were synchronized such that a specific video frame in the 2nd video camera was equivalent to the same frame time-wise in the 1st video camera,

allowing synchronizing of cheetah event timestamps with specific frames of the rat's reaching motion in the 1st video camera's view. The recording, synching, and handling of video files was automated and controlled by a BASH script. Image 3 and Image 4 show the front video camera view and side video camera views, respectively.

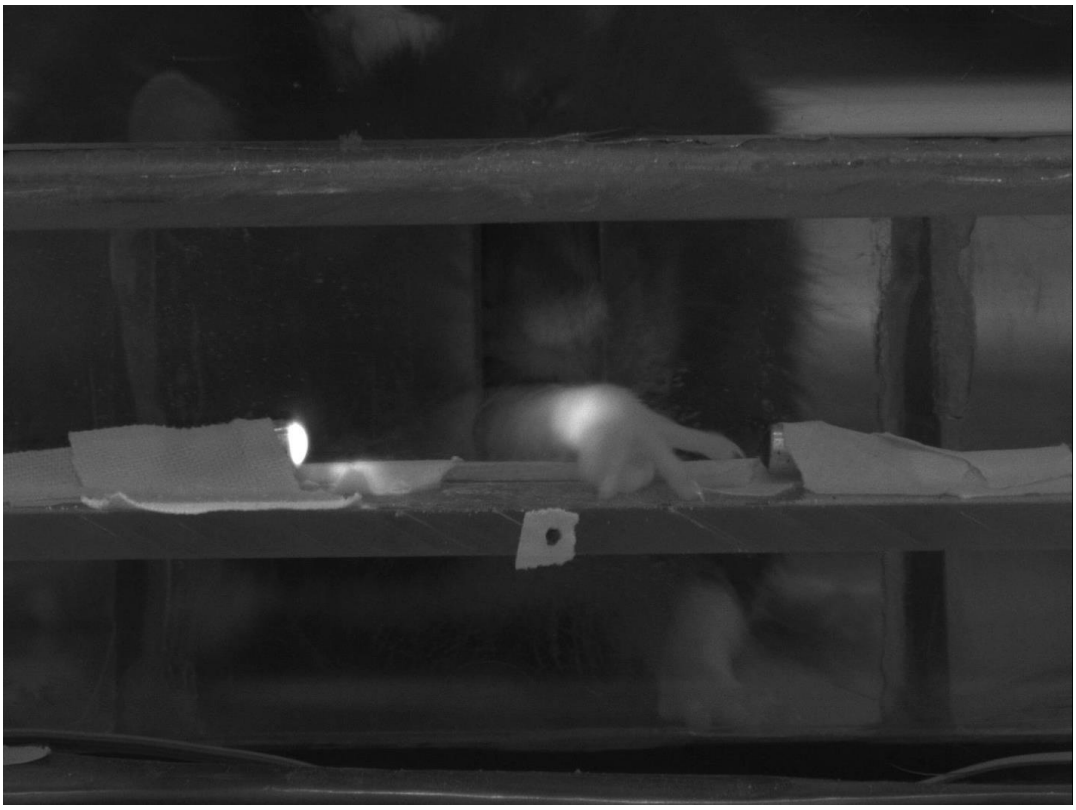

Image 3. Example frame from 1st video camera depicting a front view. This is the video camera used for detailed video reaching analysis (not in this thesis). The black dot on the white tape was used as a point of reference to account for shifting due to table vibrations. The white light shining on the paw is the infrared beam being blocked, thus triggering a beam break event as seen with a bright LED flash in Image 4. The barrier the paw is arcing over is also seen.

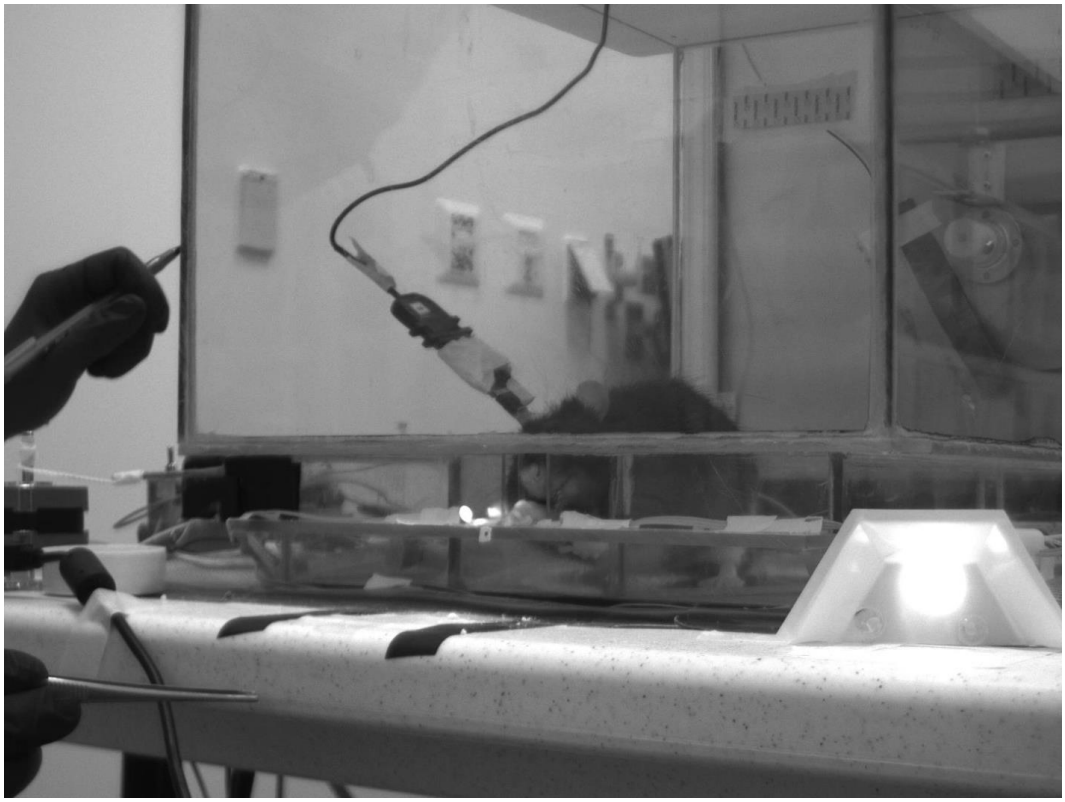

Image 4. Example frame from 2nd video camera depicting a side view. LED indictors in view at bottom right, with the middle LED signifying an infrared beam break has occurred.

2.7 Data Analysis

2.7.1 Reaching Scores

Number of successes, fails, and total reach attempts were recorded per rat per day during the Training Phase. Percent success score was computed using number of successes out of 20 total reach attempts. Group averages and variation were also computed in addition to individual plots of rat percent success scores over training days. All of these computations and visualization were done in MATLAB.

2.7.2 Video Analysis

Recorded videos of reaching attempts were sometimes used to help score ambiguous reaches. Videos were primarily for detailed limb movement analysis, based on limb coordinate labelling performed by DeepLabCut (Mathis et al., 2018), a program based on a deep neural network that assists in automatically labelling frames of a video based on supplied examples. This analysis was performed by another researcher, outside of this thesis project.

2.7.3 Electrophysiology

All analyses of electrophysiology data were conducted using MATLAB Version 2022b, with some use of libraries from Neuralynx Inc. for importing the Cheetah recording data into MATLAB. All recordings were imported to MATLAB and first separated into the three recordings epochs (pre-task rest, task, and post-task rest). Then, they were further separated into motion and motionless periods based on an accelerometer signal power threshold. Then within the motionless periods, the recording is categorized into SWS, REM or quite wakefulness (motionless period that is neither SWS or REM).

To find REM periods, the hippocampal LFP was down sampled to 100hz and then bandpass filtered to find the theta portion (5-10hz) and delta portion (1.5-4hz). The theta and delta portions of the LFP were then converted to their power signal by squaring and then smoothing the signals. A REM power ratio was computed that took into account the theta power over delta power ratio and created a threshold based on 1.5 times the standard deviation of the REM power ratio. Regions of the power signal above the threshold were counted as REM periods.

To find SWS periods, the hippocampal LFP was also down sampled to 100hz and then bandpass filtered to find the delta portion (1.5-4hz), then converted to delta power by squaring and then smoothing the signal. A threshold was created based on 1.5 times the standard deviation of the delta power signal, and any portion of the delta power signal above this threshold was deemed as SWS. Note that REM/SWS periods shorter than 10s were removed.

To compare SWS, Spindle and SWS duration differences across groups, days and epochs, one-way and factorial ANOVA's were utilized in a permutation test. To help combat the low sample size and possible violations of ANOVA statistical assumptions, the permutation version was employed. This entailed each regular ANOVA's F statistic being compared to the distribution of F statistics created by 5000 ANOVAs on shuffled data to approximate the null-hypothesis' distribution. Peri-event histograms were also created between spindles, SWRs and DBS pulses by computing the cross-correlation for each comparison per epoch, and then averaging the resulting vector across the groups of interest to compare. Because the timing vector used for comparing the events was based on the original 8000Hz sampling frequency, peri-event histogram results typically displayed a strong quantized appearance. To aid with visualization, smoothing using a 6.25ms wide window for a moving average filter was applied to the results. An example of the pre- and post-smoothing results are in Figure 4. However, for statistical testing employing multiple two-sample t-tests, the 8000Hz peri-event histogram results were down sampled to 50Hz. This down sampling helped prevent too many multiple comparisons being made, which had their alpha value corrected for using a Bonferroni correction.

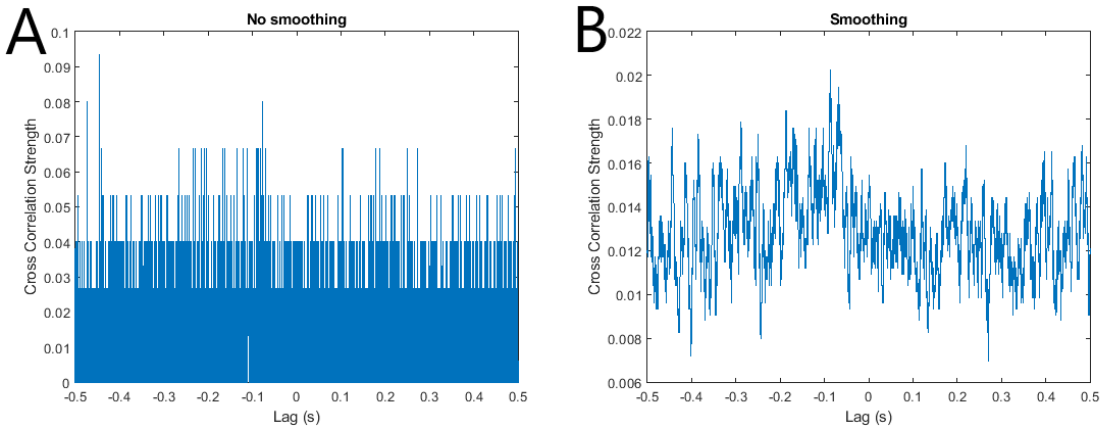

Figure 4. Example of smoothing procedure on peri-event histogram results. A.) No smoothing applied. B.) 6.25ms smoothing window applied. Both examples depict the same data, specifically the pre-task epoch peri-event histogram of spindle onset to SWR onset for all rats except rat 1E (who had no suitable channel for SWR detection).

Spindle detection

A single candidate cortical LFP channel was chosen from each rat for spindle detection quality based on visual inspection. Cortex LFP signals during motionless periods were bandpass filtered between 10-20Hz, and then converted to a power signal by squaring and then smoothing the signal. Regions in the spindle power signal above a threshold of 1.5 standard deviation of the signal were classified as spindles. Any spindle less than 200ms or longer than 2s were removed.

DBS pulses created large spikes in voltage, and this was frequently mistaken as spindles by the detection algorithm. To counter this, a similar method as employed by Eckert et al. (2021) was used for stimulation artifact removal before spindle detection. Specifically, an inverted Blackman window of roughly 8ms wide centered around the voltage peak was applied. Then the signal was smoothed with a 2.5ms window starting

from 6ms before the previous voltage peak, and until about 113ms after the voltage peak, but applied with a linearly decreasing gradient such that smoothing was most aggressive near the voltage peak and weakened towards the edge of the smoothing window. Smoothing window size was determined through visual inspection of when spindle frequency power typically returned to normal levels after a stimulation event.

After regular spindle detection was performed, spindles were further subdivided into endogenous spindles and DBS evoked spindles. DBS evoked spindles were defined as spindles that started between 90-300ms after a DBS pulse was delivered. This range was based on the significant region of increased spindle events after a DBS pulse, as reported in section 3.2 Electrophysiology Results and shown in Figure 12. Spindles that did not begin in this region were thus considered endogenous spindles. Control rats did not receive DBS, so all spindles detected were considered endogenous spindles. The rate of these evoked versus endogenous spindles were normalized per time in SWS and then compared across epochs for both groups. For post-task rest specifically, different durations of rest from the start of the epoch were also compared for the rate of evoked and endogenous spindles found per minute of SWS.

SWR detection

A single candidate hippocampal LFP channel was chosen from each rat for SWR detection quality based on visual inspection. To detect SWRs from a hippocampal channel, LFP signals from motionless periods were bandpass filtered between 100 and 300Hz, converted to a power signal via squaring and then smoothed. Regions in the SWR power signal that were above 3 standard deviations of the signal were classified as

SWRs. Any SWRs shorter than 15ms or longer than 150ms were also removed. These removal boundaries are based on Eckert et al. (2021) and Liu et al. (2022).

The DBS stimulation evoked a response in the hippocampal LFP that was mistakenly detected as SWRs, so detected SWRs within +/- 50ms of a voltage peak due to stimulation were removed as well. This removal boundary was also determined from a visual inspection of when SWR frequency power typically returned to normal after a stimulation event. This removal of SWR overlapping with DBS is also reflected in Figure 13 in section 3.2 Electrophysiology Results.

Figure 5 shows an example portion of a recording showing detected spindle and SWR events.

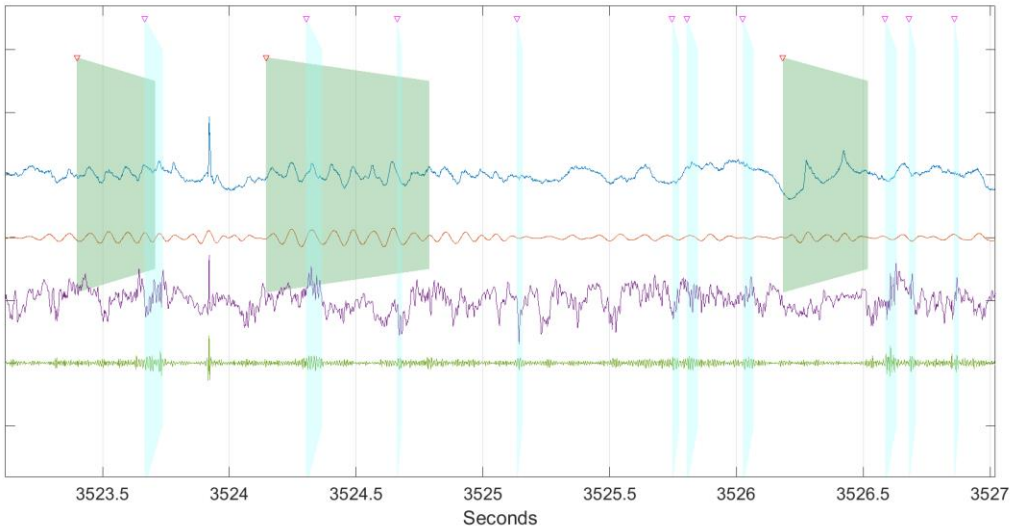

Figure 5. Example of detected spindle and SWR events. There are four LFP traces, from top to bottom: blue is a raw cortical LFP, orange is a 10-20Hz band passed cortical LFP, purple is a raw hippocampal LFP, and green is a 100-300Hz band passed hippocampal LFP. Green trapezoids show detected spindle events. Blue trapezoids show detected

SWR events. Note that the spike in voltage just before 3524 second mark is an example of a DBS stimulation event.

3. Results

3.1 Behavioural Results

The results of the single-pellet reaching task are presented in Figure 6. A 2 x 15 factorial ANOVA was run through a 5000 shuffle permutation test, and found no statistically significant differences as a function of group (F(1,88) = [.34], p = .54) or day (F(14,56) = [1.73], p = .07). No interaction was found either (F(14,56) = [.42], p = .96).

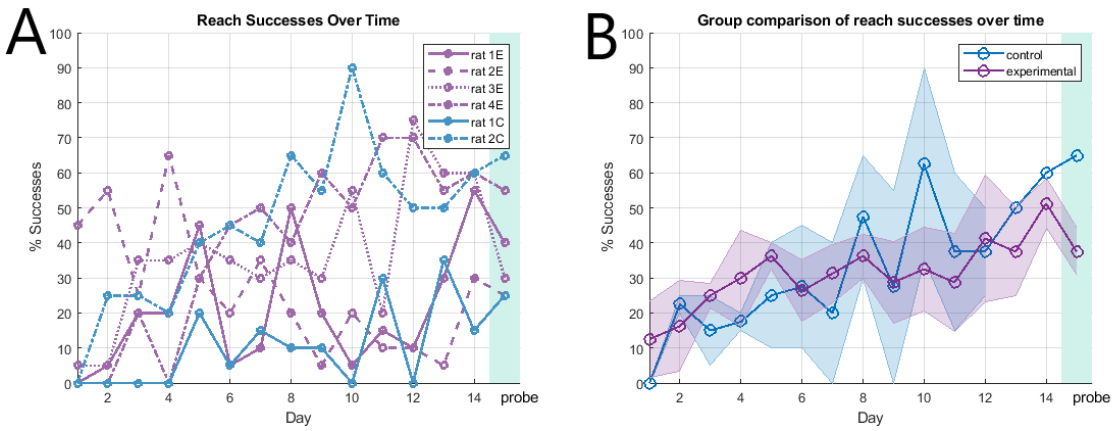

Figure 6. Single-pellet reaching task results. A.) Individual performance results of each rat. B.) Group averages of reaching performance. Shaded regions behind traces represents SEM. Note that control group error bar ends on day 12 due to a 3 day leftwards shift in performance of rat 1C due to not understanding the task until day 4 which is the day that preceded its first learning success.

3.2 Electrophysiology Results

Local field potential data were analyzed, and the resulting properties are presented. An example of an epoch's sleep stage categorization and stimulation results is shown in Figure 7.

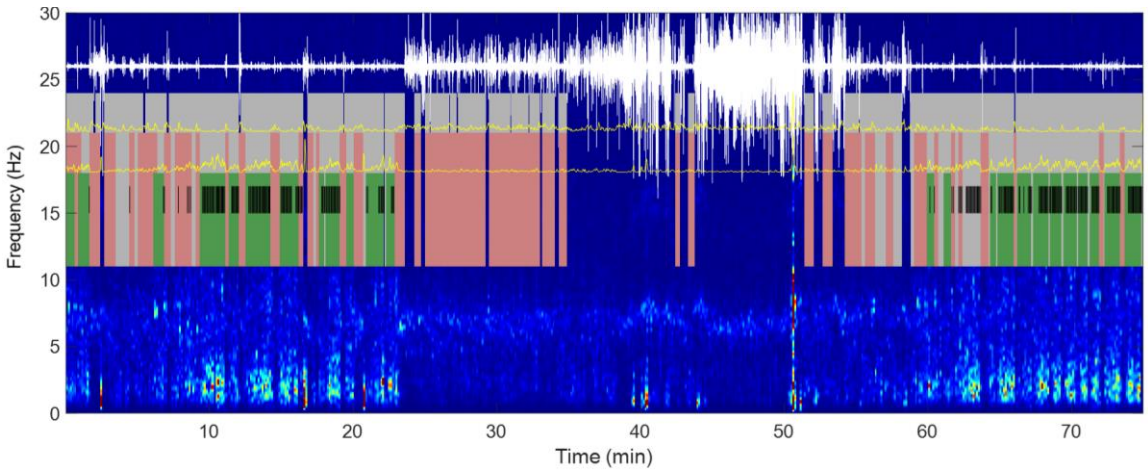

Figure 7. Example of sleep architecture. Shown from a post-task rest epoch from rat 4E on day 3 of training. The interpretation of this figure is similar to those used in figure 3b in Eckert et al. (2020). The top white trace represents the accelerometer signal which was used to divide motion and motionless periods (grey boxes). Red boxes represent REM and green boxes represent SWS. top and bottom yellow traces represent the computed and smoothed power of the power ratios for REM and SWS, respectively. Spectrogram behind boxes is from a hippocampal channel used for sleep stage detection. Lastly, the vertical black ticks represent DBS and TMR delivery.

After sleep stage classification, spindle detection and SWR detection, the occurrence and summary of these measures can be found in Figures 8 through 10. Figure 8 shows SWS duration across pre- and post-task rest epochs. A one-way ANOVA was run through a 5000 shuffle permutation test on the mean percent time in pre-task SWS charts, comparing the main effect of days. It found no statistically significant difference as a function of day ($F(14,56) = [.55]$, $p = .90$).

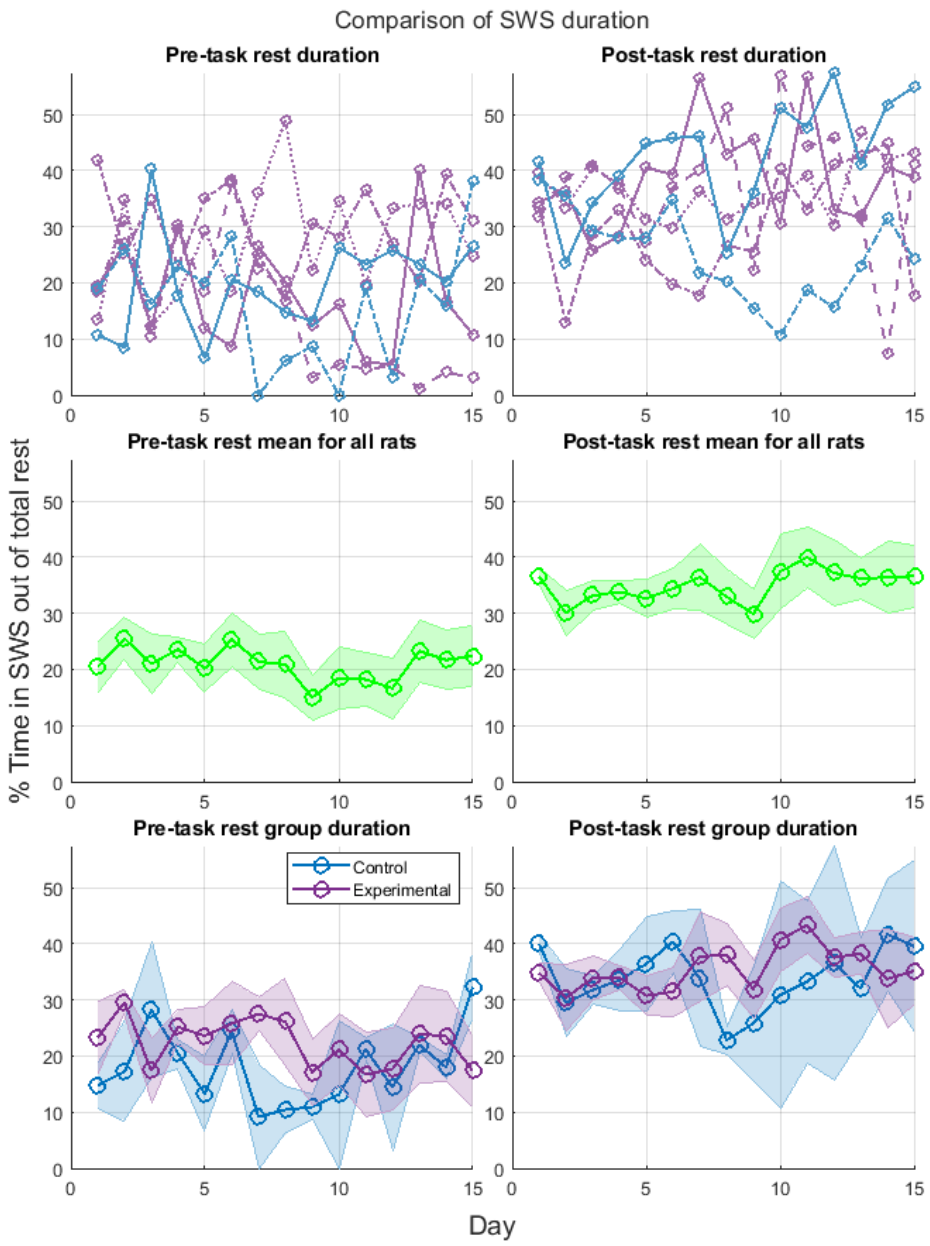

Figure 8. Summary of SWS results. Left-hand side figures are for pre-task rest epoch and right-hand side figures are for post-task rest epoch. Due to space constraints, the legend for the top two figures was omitted, but uses the same color and pattern scheme as figure 6A in section 3.1 Behavioural Results. All shaded regions behind traces represent SEM. Day 15 represents probe trial.

A 2 x 15 factorial ANOVA was run through a 5000 shuffle permutation test and found that pre-task rest epochs did not differ in the percent time spent in SWS during the epoch as a function of either group (F(1,88) = [3.09], p = .08) or day (F(14,56) = [.55], p = .90). No interaction effect was found either (F(14,56) = [1.03], p = .44). The same test was performed for the post-task rest epoch results and found no statistically significant differences from group (F(1,88) = [.42], p = .51) or day (F(14,56) = [.48], p = .93). Again, no interaction effect was found either (F(14,56) = [.67], p = .80).

Figure 9 shows the spindle event results. A 2x15 factorial ANOVA was run through a 5000 shuffle permutation test on the mean spindles per minutes charts across pre- and post-task rest epochs, comparing the main effect of group and days. For pre-task epochs, there was no statistically significant difference as a function of day (F(14,56) = [.55], p = .82), or group (F(1,88) = [3.35], p = .06). No interaction effect was found (F(14,56) = [.40], p = .92). Note rat 2E exhibited a few days of spindles per minute of SWS counts that were clear outliers, and this was due to an under classification of SWS during certain days, which may be due to a recording malfunction. All above tests for pre-task epochs show the results with rat 2E included but excluding it did not change the p-values into significant differences for main effect of group (p = .11) or interaction (p = .69). However, for main effect of day the exclusion of rat 2E changed the significance to p = .03.

Performing the same permutation ANOVA test for comparing the main effect of group and day in post-task rest, it was found that there was a statistically significant main effect of group (F(1,88) = [5.62], p = .02), but not day (F(14,56) = [.99], p = .47). No interaction effect was found (F(14,56) = [.84], p = .62).

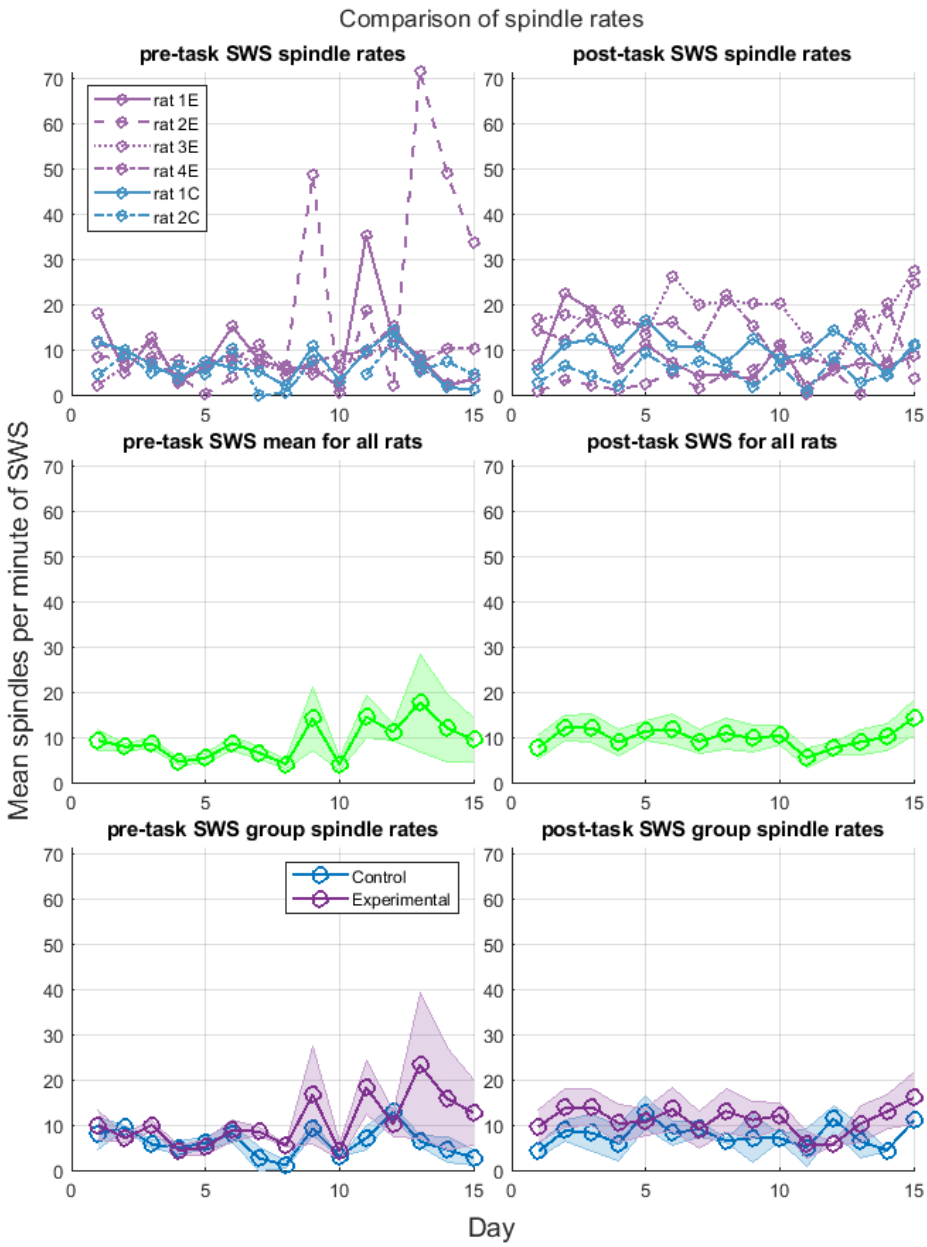

Figure 9. Summary of spindle results. Left-hand side figures are for pre-task rest epoch and right-hand side figures are for post-task rest epoch. All shaded regions behind traces represent SEM. Day 15 represents probe trial.

Figure 10 shows the SWR event results in a similar format to the spindle results in Figure 9. Note that rat 1E was removed from the SWR analysis due to having no

adequate hippocampal channels for SWR detection. However, rat 1E still had SWR detection conducted on the most ideal channel and the results are included in Figure 10. Using the same ANOVA design for the spindle tests, it was found that there was no statistically significant difference due to the main effect of day ($F(14,56) = [.51]$, $p = .89$) on mean SWR's per minute during pre-task epoch SWS. There was also no main effect due to group ($F(1,88) = [.86]$, $p = .36$) or interaction ($F(14,56) = [.33]$, $p = .98$). For post-task epochs, it was found that there was no difference due to main effect of day ($F(14,56) = [.79]$, $p = .64$) or group ($F(1,88) = [.14]$, $p = .77$). There was again no interaction as well ($F(14,56) = [.27]$, $p = .99$).

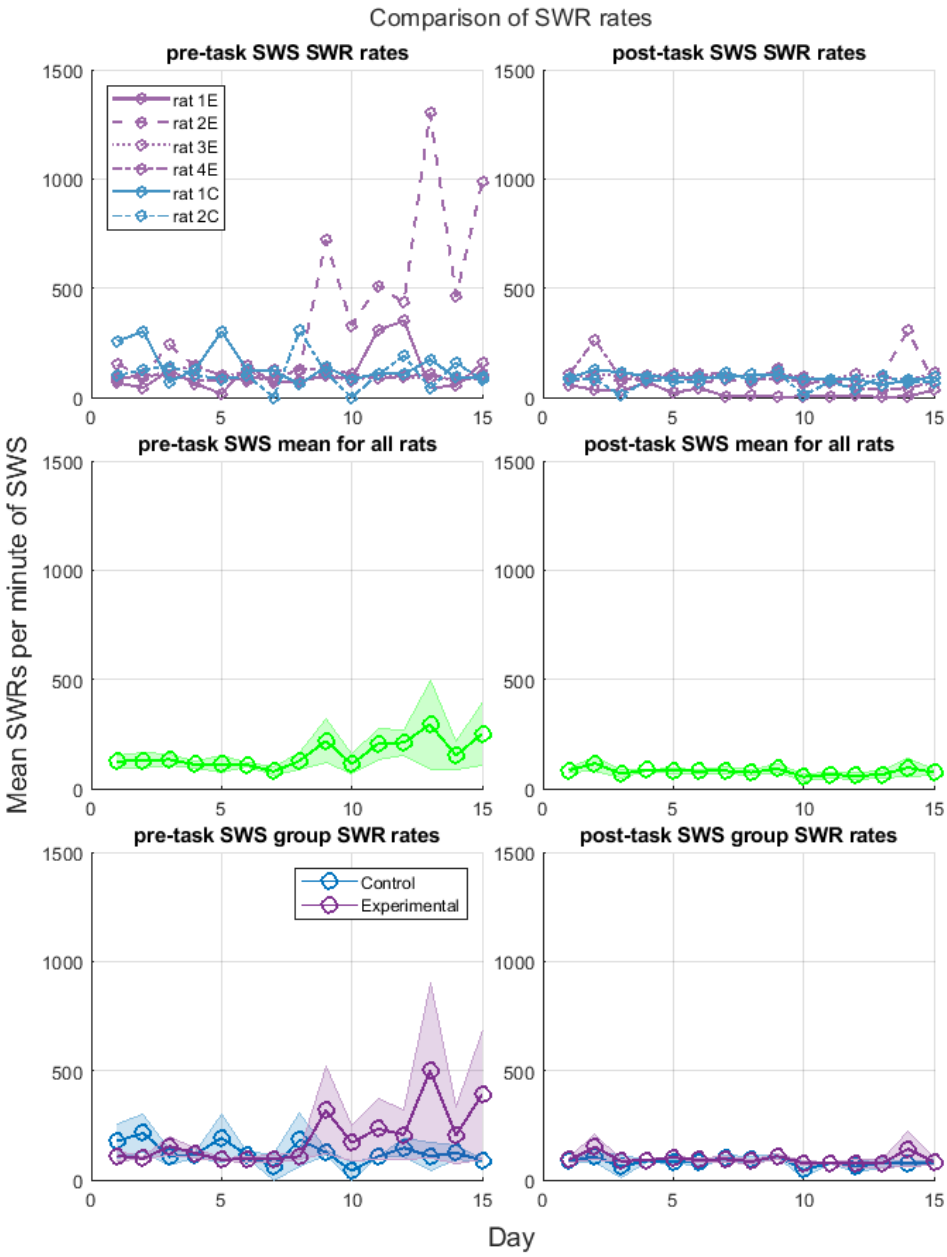

Figure 10. Summary of SWR results. Left-hand side figures are for pre-task rest epoch and right-hand side figures are for post-task rest epoch. All shaded regions behind traces represent SEM. Day 15 represents probe trial.

Moving on to per-event histogram results: peri-event histograms were created between spindle and SWR onsets, and also with each event relative to DBS pulse events.

These results are shown in Figures 11-13. Comparing versus a histogram of the shuffled events, a series of two-sample t-tests with Bonferroni correction were conducted and found that there were lag periods with statistically significant differences with the spindle onset to SWR onset peri-event histogram versus shuffled control ($p<9.8\times10^{-4}$) (Figure 11). The same test was conducted for the DBS pulse peri-event histogram results, comparing the experimental group to control group's peri-event histogram (noting that Rat 1E was removed due to having no suitable channels for SWR detection). It was found that statistically significant differences with the spindle onset to DBS pulse peri-event histogram existed at time lags in the approximate 90-300ms range after a DBS pulse ($p<9.8\times10^{-4}$) (Figure 12). No Statistically significant differences were found for the SWR onset to DBS pulse peri-event histogram results between groups ($p>9.8\times10^{-4}$) (Figure 13).

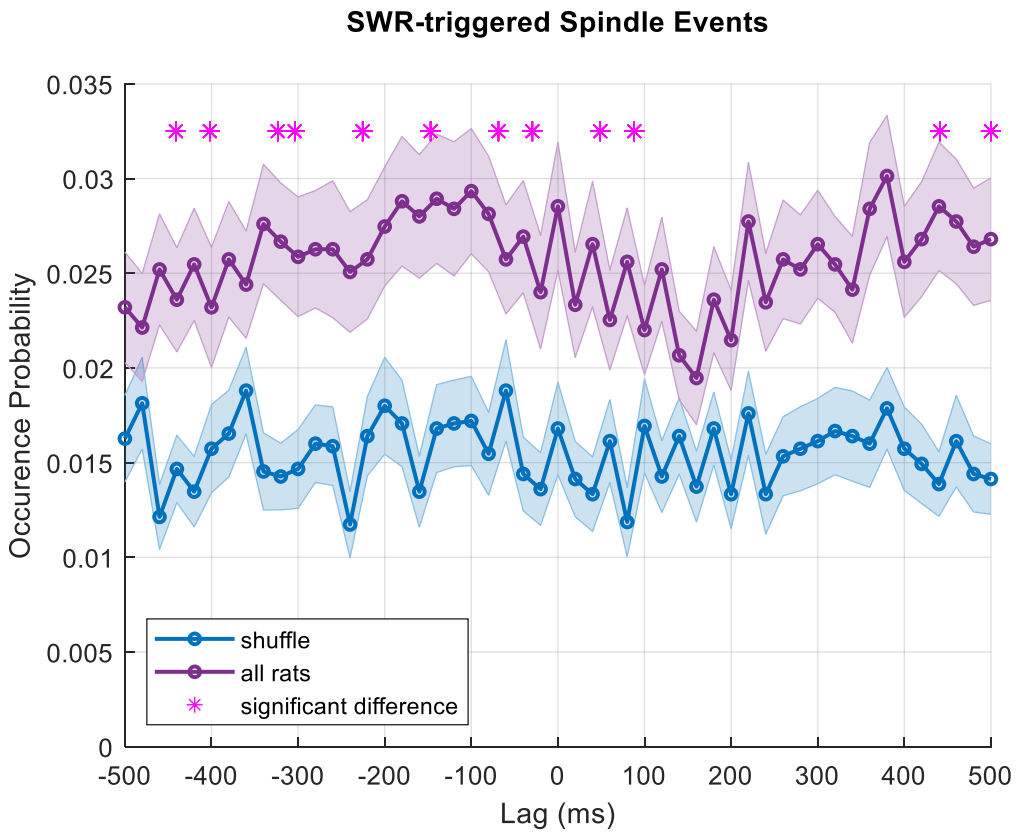

Figure 11. Peri-event histogram of spindle onset relative to SWR onset. Lag of 0ms represents time of SWR onset. Shaded regions behind traces represent SEM. Pink asterisks (*) represent time lags with a significant difference versus a shuffled control.

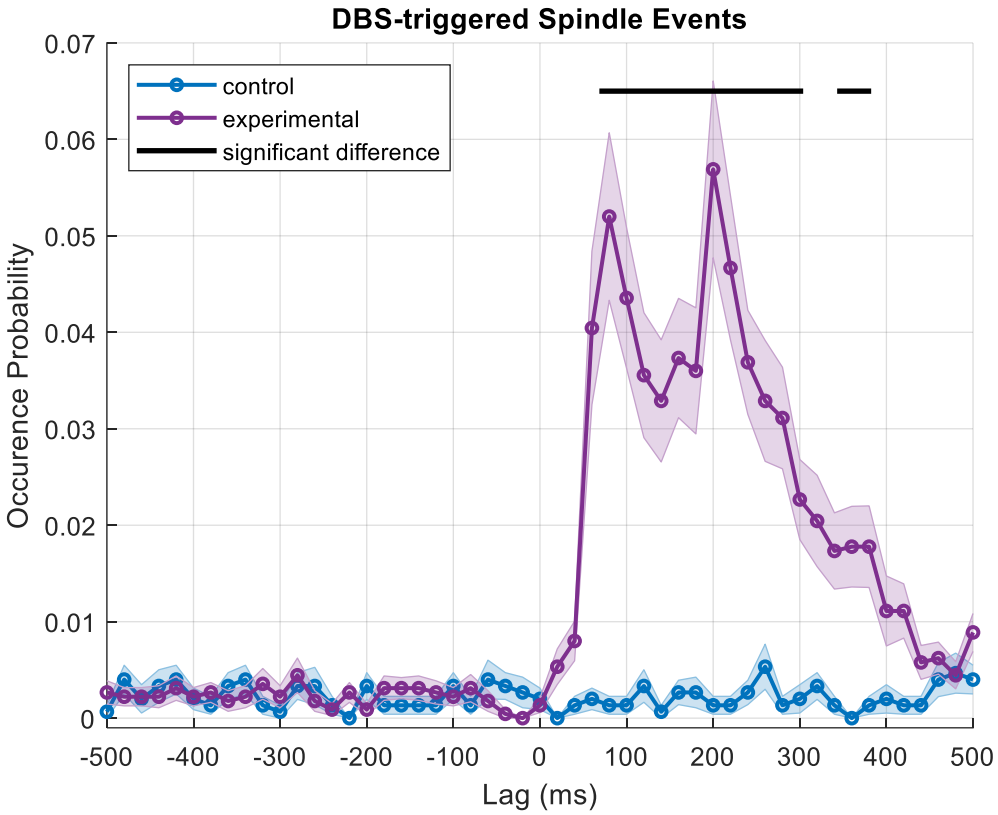

Figure 12. Peri-event histogram of spindle onset relative to DBS onset. Lag of 0ms represents time of DBS onset. Shaded regions behind traces represent SEM. Black bars represent time lags with a significant difference between groups.

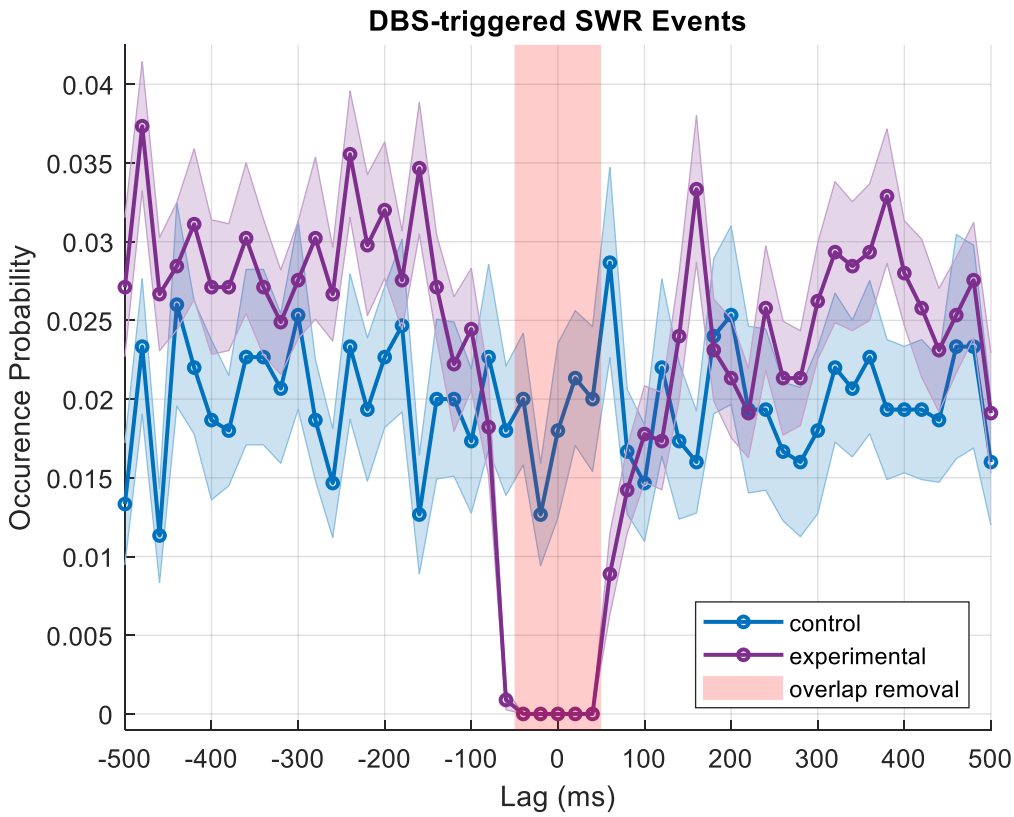

Figure 13. Peri-event histogram of SWR onset relative to DBS onset. Lag of 0ms represents time of DBS onset. Shaded regions behind traces represent SEM. Red overlap region shows the +/-50ms region around DBS events where SWR events were removed during preprocessing. Therefore, SWR probability is zero within this region in the peri-event histogram. While excluding the overlap removal region, no significantly different lags were found between groups.

Lastly, the comparison of endogenous and DBS evoked spindles. These results are shown in Figure 14 and Figure 15 below. For the pre-task rest epoch in Figure 14, the high endogenous spindle rates seen on rat 2E for days 9,13,14 and 15, and for rat 1E on day 11 were removed for this analysis based on the justification of being greater than approximately 2 standard deviations from the mean. The removal of these outliers

weakens any spindle rate differences found between groups. This outlier removal would also not affect a within experimental group comparison because the outliers are due to an under classification of SWS, and not due to disproportional classification of endogenous and/or evoked spindles.

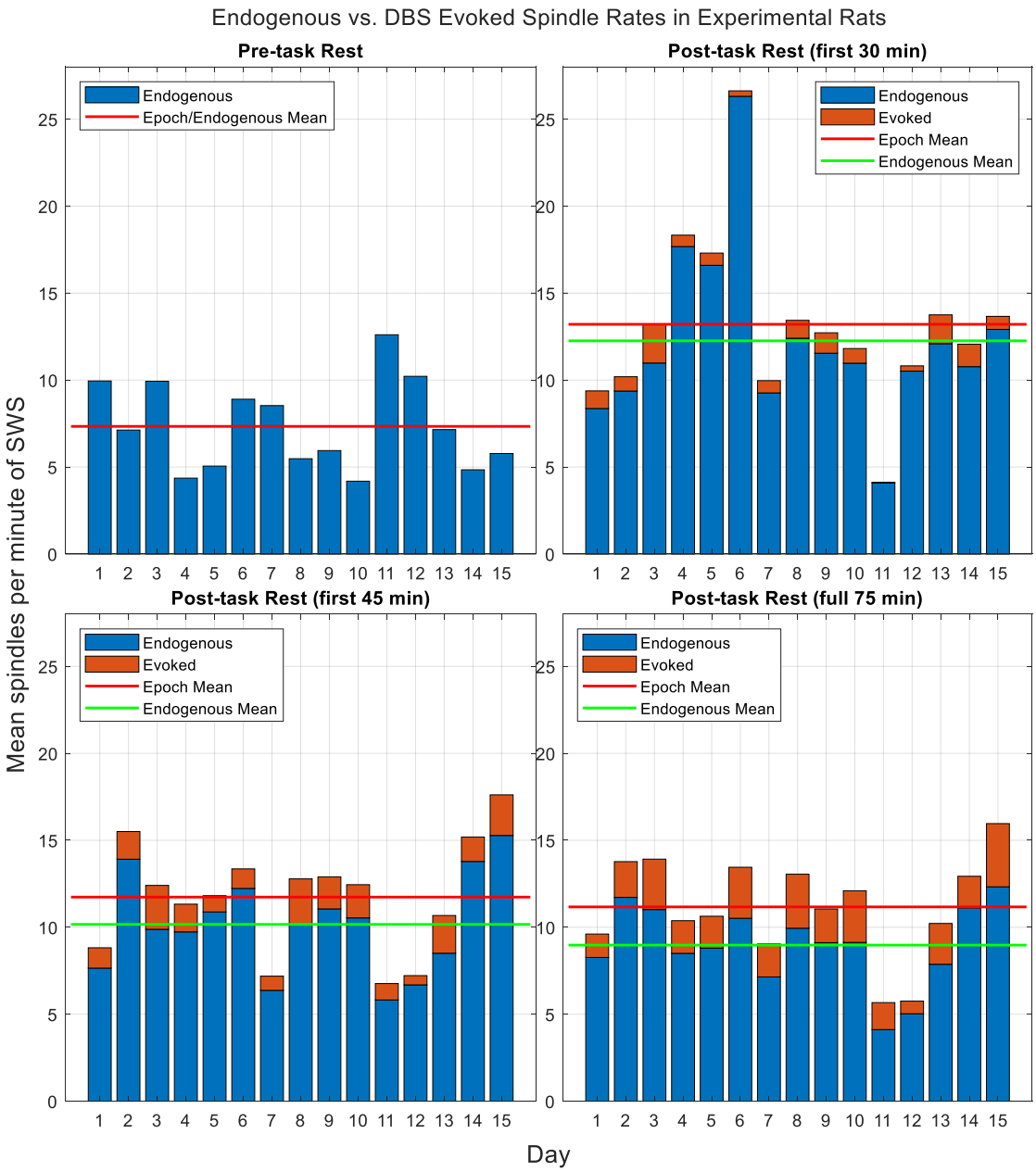

Figure 14. Endogenous vs. DBS evoked spindle rates in experimental rats. Top left chart depicts pre-task rest epoch, while other figures show different durations of the post-task rest epoch. Epoch mean was calculated from the total spindle rate across days (seen as the total bar height). Endogenous mean was calculated using only the endogenous counts across days (seen as the height of the blue bars). Day 15 represents probe trial.

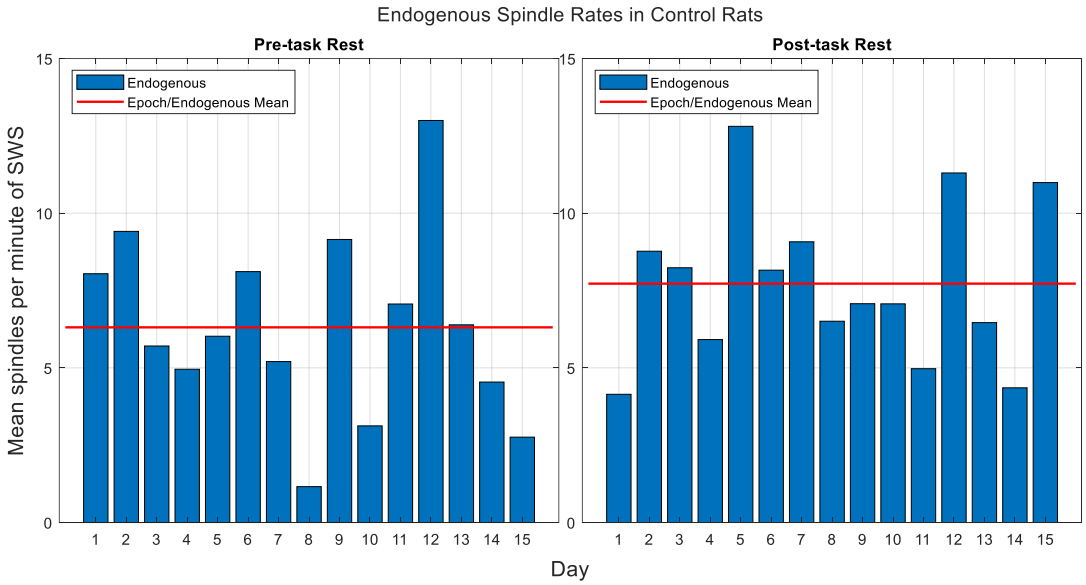

Figure 15. Endogenous spindle rates in control rats. Due to having no DBS evoked spindles in control rats, the epoch mean for spindle rates is identical to the endogenous spindle rate mean. Right figure is from the post-task rest's full duration of 75 minutes. Day 15 represents probe trial.

For the experimental rats in Figure 14, a Wilcoxon Rank Sum test was performed to compare both epoch mean and endogenous mean spindle counts between pre-task rest and the three post-task rest epochs, resulting in 6 comparisons total. The resulting p-values are shown in Table 4 below. For the control rats, A Wilcoxon Rank Sum test was also performed comparing pre-task rest epoch/endogenous spindle rates to post-task rest

at the same three durations as with the experimental group (only the full 75 minute duration results are shown in the right side of Figure 15 though), resulting in 3 comparisons in total. The resulting p-values for the 75 minute, 45 minute and 30 minute comparisons were $p = .31$, $p = .83$, $p = .69$, respectively.

Pre-task rest vs post-task rest of length:	Total spindle rate	Endogenous spindle rate
First 30 min	$p = .04$	$p = .17$
First 45 min	$p = .05$	$p = .23$
Full 75 min	$p = .16$	$p = .81$

Table 4. Summary of p-values for total and endogenous spindle rate comparisons.

4. Follow-up Experiment

4.1 Experiment Redesign Justification

Section 3 Results details the findings based on only a single cohort of 6 rats. The original plan was to test 24 rats in total. However, the interim results from the first 6 rats were not promising as evident in the lack of any emerging group differences in reaching performance (see Figure 6A). In addition to the negative statistical finding of no group differences so far, a power analysis was conducted based on the reaching results from the 6 rats and their variances, using G*Power (Faul et al., 2009). Assuming a generous 20% increase in performance in the experimental group versus controls, and conducting a repeated measure ANOVA, it would require approximately 80 rats total to obtain 0.8 power to find an effect. Therefore, it was concluded that continuing the original experiment as planned was not practical under the current design.

In light of the interim findings and resulting power analysis, the main experiment was ended. However, using all of the observations and work of the main experiment, a follow-up experiment was planned to improve the design further while still testing the question of whether motor learning could be enhanced. The two aspects of the previous experiment I wanted to address were about not understanding what part of the reaching task was novel motor learning, and the observation that some rats had varying levels of understanding the nature of the task during early training. These two issues are discussed in more detail in section 5.2 Limitations. The new proposed experiment would be a similar 2 group single-pellet reaching task with 24 rats in total. The new component would be two phases in the training of the task. The first 14 days of the task would be with the regular reaching task, training the rats to achieve asymptotic performance and ensuring they understand the task. Then in phase 2, the task will switch to a more difficult variation of the single-pellet reaching task for 14 days. This switch part way through training ensures that all rats understand the basic task, and controls for what the new motor learning aspect is through the difference in skill needed to reach in the new variation. However, in order to conduct this proposed experiment, a few details needed to be confirmed via pilot studies.

4.2 1st Pilot

The first pilot study was to test different possible variations of the single-pellet reaching task to test their suitability as a more difficult but also learnable task. Ideally, this would be demonstrated by a drop in reaching success on the first day of phase 2, but then a return to higher asymptotic performance afterwards as training continued. Such a task may then be a suitable test of showing differences in motor learning. Two main

variations were tested: a taller barrier, and a lowered shelf. For the taller barrier, the height was doubled to 5mm tall, with two locations of the pellet well tested: normal distance ("close-well") and a further distance ("far-well"). For the lowered shelf variation, varying heights of a lowered shelf were tested, measured in millimeters lowered from the original height. Five rats from after the main experiment was conducted were used for this pilot. These multiple variations were tested in succession after an initial 3 days of retraining on the original task to have recent baseline performance to compare to. One rat dropped out after day 17 due to a health complication. Results of this pilot are shown in Figure 16 below.

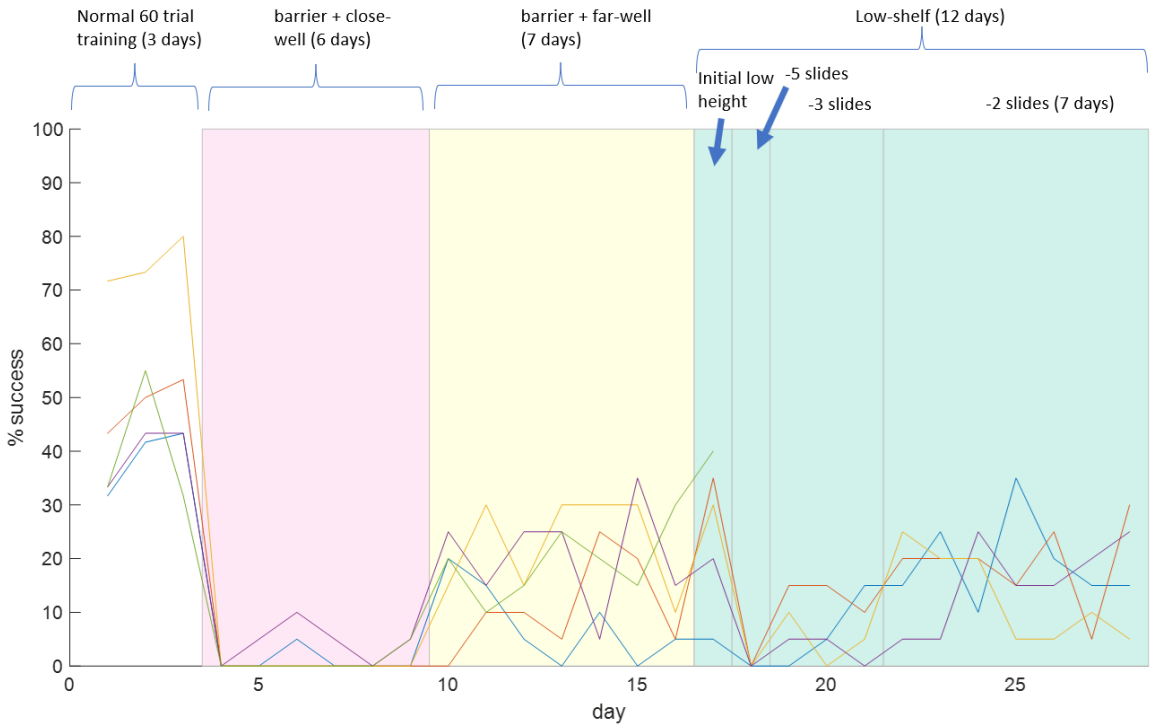

Figure 16. Reaching success across variations of the single-pellet reaching task. Note that every variation entailed 20 trials per day except for the first normal training period for 3

days which had 60 trials per day. For the low-shelf variations, 1 slide is equivalent to 1mm.

It was found that during the testing period, all test variations were more difficult than the original task, but none exhibited increased performance over time suggesting learning. Specifically, the tall barrier plus far well combination and -2mm low shelf variation were both tested for 7 consecutive days as the best variation candidates. Fitting a linear trend line to both variation's data also confirmed a lack of a nontrivial positive slope (slope = .107 and -.179 for tall barrier plus far well and -2mm low shelf variations, respectively).

4.3 2nd Pilot

The 2nd pilot was to test whether the pellet on the lower shelf is visible/sensible to the rats, possibly explaining the poor performance in the previous pilot. To test this, the remaining 4 rats from the 1st pilot were tested on the normal reaching task for 4 days. Each day, each rat went through 60 reaching trials. However, half of the reaching trials had no pellet placed, but the rest of the setup and actions of the experimenter were the same. The no pellet trials were randomly distributed throughout the total 60 trials, and the order was re-randomized each day. It was predicted that if the rat cannot sense/see the pellet, then the amount of reaching attempts will decrease during trials with no pellet versus trials with a pellet. The results of this experiment are in Table 5 and Table 6.

Day	Rat 1E	Rat 2E	Rat 3E	Rat 2C
1	29	30	9	15
2	29	30	29	30

3	27	30	30	30
4	30	30	30	30

Table 5. Number of pellet reach attempts. Each cell is out of a maximum score of 30.

Day	Rat 1E	Rat 2E	Rat 3E	Rat 2C
1	28	30	11	16
2	30	30	27	30
3	27	30	29	30
4	30	30	30	30

Table 6. Number of no pellet reaches. Each cell is also out of a maximum score of 30. Note that not every cell when summed with its respective cell from Table 5 equals 60 because not every rat completed 60 trials, particularly on early days when signs of stress or prolonged distraction caused an early stop to the trials.

A Chi-square test was conducted on the counts and found no significant difference in the rate of reach attempts when a pellet was present versus no pellet present, X^2 (1,907) = .78, p = .38.

5. Discussion

5.1 Results Summary and Interpretation

For the behavioural analysis, no statistically significant difference was found in reaching performance between the experimental and control group. For the electrophysiological analysis, it was confirmed that the experimental group did have an increased rate of spindle events during post-task rest versus the control group. Main effect of day did significantly predict the spindle rate during SWS during the pre-task epoch. Other sleep measures did not differ between the groups or days. For peri-event histograms, both spindle to SWR and spindle to DBS found specific lags that were significantly different from their shuffled control or control group. For the endogenous and DBS evoked spindle comparisons, it was found that for experimental rats the total spindle rate increased during the first 30 minutes and first 45 minutes in post-task rest, but not for any other comparisons. For the 1st follow-up experiment, no variation of the single-pellet reaching task found strong evidence for being initially difficult, but learnable over time. The 2nd follow-up experiment found no statistically significant difference between number of reaches made when a pellet was placed versus no pellet placed in a test of pellet sensing.

Regarding interpretation of the main experiment's reaching results, an initial conclusion can be made that DBS and TMR did not improve the rate or peak performance in the reaching task. It is unfortunate however, due to the nature of LFP recording, that the efficacy of TMR in increasing memory reactivation of the reaching task probably cannot be confirmed without unit activity. Without unit activity data, it is

difficult to predict whether the lack of effect was due in part to TMR being ineffective or not because this would require confirming if TMR did induce a change in neural activity, ideally through memory reactivation. For DBS, despite confirming that there was an increase in spindle events in the experimental group, this did not result in an increase in learning performance (or at least one that is statistically detectable). However, I will later argue (section 4.2 Limitations) that there are some limitations that may prevent a conclusion about the efficacy of DBS, in addition to TMR, for this experiment. Nonetheless, if DBS was found to not be effective in improving rodent motor learning, this may corroborate findings in human research for the similar technique of TES outlined in a meta-analysis (Barham et al., 2016) which found no effect for TES delivery on procedural tasks despite efficacy for declarative tasks.

An important distinction should be made between memory acquisition and memory retention, with the former being largely focussed on in this work so far. Pan and Rickard (2015) argue in their meta-analysis that procedural memories are not enhanced during sleep but rather stabilized, and that performance gains are the consequence of online practise and the progression of time. With that viewpoint in mind, the probe trial results which take place a week after day 14 of training may act as a measure of memory retention as opposed to acquisition. With only one day of data, it is not feasible to conduct a properly powered statistical analysis of whether it likely differs from the performance towards the end of regular training. An informal examination of the group means during the probe trial using a Wilcoxon rank sum produces a $p = .93$ for a group difference, but this is based on only a total of 6 data points. Nonetheless, considering the high p-value and visually extrapolating a learning trendline from the later days of training

to the probe trial, there is moderate evidence that the groups did not differ in performance during the probe trial. Thus, this is weak evidence that the retention of the task is comparable between the groups, similar to a lack of difference in the acquisition of the task. However, future work should perhaps entail a longer probe trial period with a larger sample to more conclusively test for retention of the task.

However, learning performance aside, it was confirmed that the DBS protocol did enhance the rate of spindles during SWS in the experimental group versus the control group in the post-task epoch. This provides additional evidence for the efficacy of the DBS protocol proposed in Eckert et al. (2021). Peri-event histogram of spindle onset to DBS pulse events (Figure 12) showing an increase around 100-300ms afterwards also adds support for the effect of Eckert et al. (2021) seeing a peak in spindle rate increase at 200ms after stimulation onset. The additional peak at around 100ms is novel versus what is report by Eckert et al. (2021), but this peak persisted through different variations of artifact removal techniques and windows, suggesting it itself is not an artifact. SWR to DBS peri-event histogram (Figure 13) was technically significant in the approximate +/- 50ms region around DBS onset. However, this exactly coincides with the window for removing SWR events occurring near DBS events during preprocessing. Therefore, this statistically significant region is only due to the SWR removal during preprocessing. Ignoring this region, there are no time lags that significantly differed.

Regarding the significant effect of day on pre-task epoch spindle rates during SWS (Figure 9): it is odd that the p-value shifted dramatically from 0.83 to 0.03 with the exclusion of rat 2E who had outlier data. Nonetheless, if you correlate the spindle data for each pair-wise combination of the remaining 5 rats, the average correlation is .35.

Performing a similar shuffle test as with the ANOVA, but on the distribution of correlation values instead of F-values, the resulting p-value for the significance of the average correlation found is .01. This can be interpreted as the average pair-wise correlation of .35 on the pre-task spindles rates during SWS is approximately greater than 99% of possible combination's resulting correlations, thus this is a relatively strong correlation. However, the interpretation of the meaning of this is not clear. Figure 17 shows the spindles rates with rat 2E removed. There is no clear increasing or decreasing trend across days, but it could be argued that some days such as 4,8 and 12 exhibit strong shared increasing or decreasing trends across all rats. The use of two recording rooms is unlikely to be the cause of this finding because half of all pre-task rest recordings occurred in each room for each day, therefore it cannot be a confounding factor influencing the effect of day on spindle rates. This result may still be the result of statistical artifacts or improper coding of the test, but nonetheless should be confirmed and investigated further.

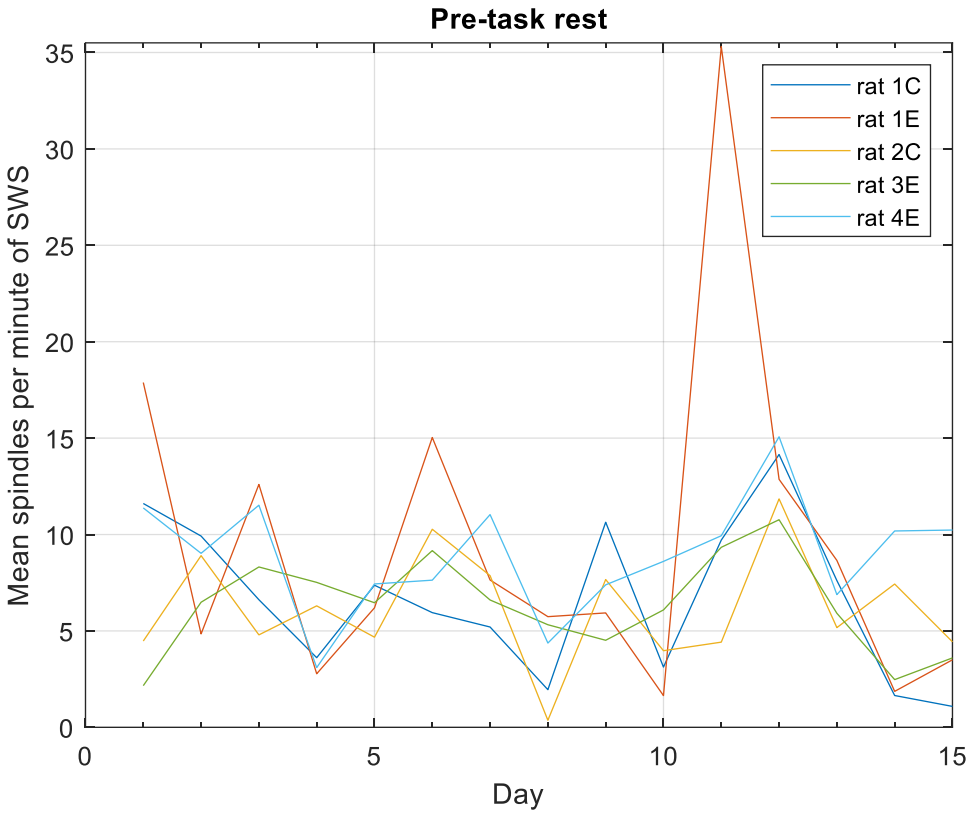

Figure 17. Spindle rates in pre-task SWS without rat 2E. This is the same data presented in the upper left subfigure of Figure 9 but excluding rat 2E to more clearly see the other data. Day 15 represents probe trial.

The spindle onset to SWR onset peri-event histogram (Figure 11) found a few scattered time lags that differed versus the shuffled control, putatively suggesting that at multiple lags that spindles and SWRs are cross-correlated in a small 1 second window. However, using a larger 20 second window shows a peculiar trend of the all-rat SWR probability gradually increasing after DBS onset (see Figure 18). Considering that SWRs are frequent (a little greater than 1 Hz during SWS), it should not be possible for spindle events to continually increase in frequency after SWRs in general beyond a short time

window, let alone a 20 second window. These results should therefore be interpreted with caution.

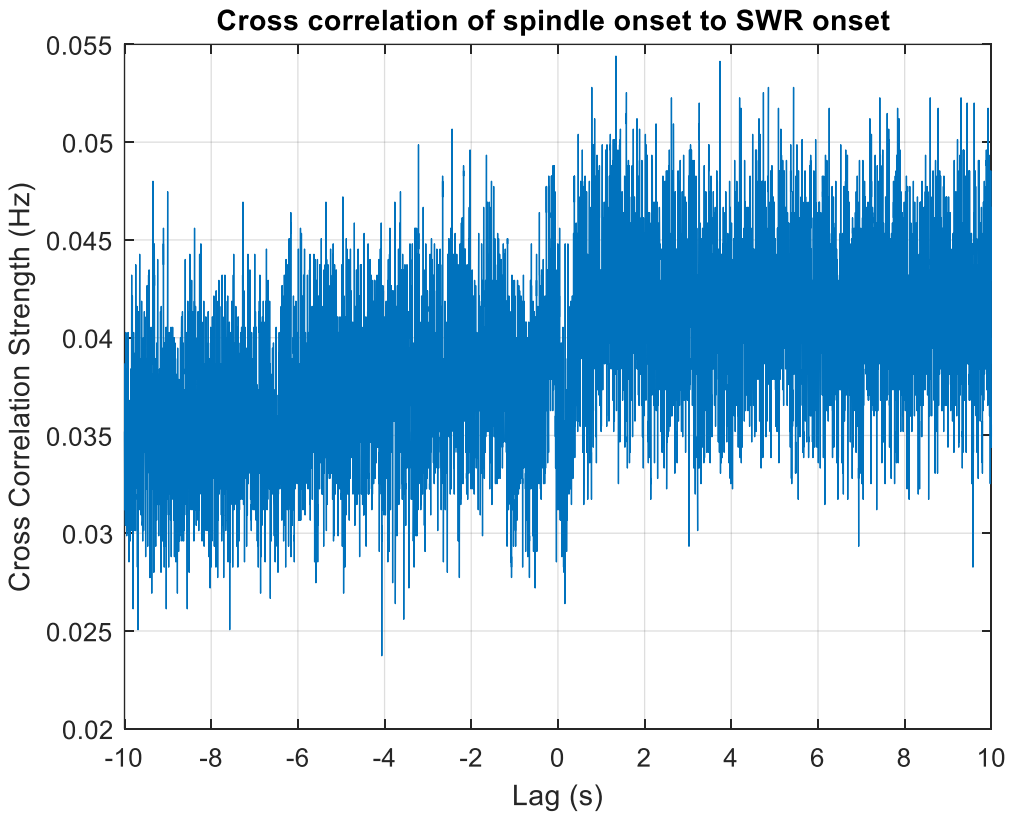

Figure 18. Peri-event histogram of spindle onset versus SWR onset with larger window. An alternate version to the data in Figure 11, now with a 20 second window and without shuffled control. Lag of 0ms represents time of DBS onset.

Regarding the endogenous and evoked spindle rate comparisons, it is important to note the issue of multiple comparisons. If multiple comparisons were properly accounted for, none of the 9 Wilcoxon rank sum tests would have elicited a significant p-value. However, I argue that the message of these results lies in the trend seen in Table 4 as opposed to whether individual p-values are significant or not. Namely, for both the total spindle rate and endogenous spindle rate columns, we see a decreasing p-value as the

duration decreases and is focused towards the beginning of rest. It is thought that memory reactivation and thus perhaps spindle events based on learning favour early sleep versus later sleep. Therefore, these decreasing p-values give evidence for that theory. On the contrary, there is no clear decreasing trend in p-values for the control rats, with no individual measure close to significance before multiple corrections either. This may be due in part to having only 2 control rats though, thus likely under-powered to find an expected effect.

Separate from the statistical results in Table 4, it is interesting to note a potential pattern in Figure 14 and the depicting averages. Having already looked at how pre-task rest compares with the red epoch mean average line and the green endogenous mean line individually, it can be argued that the difference between these two averages grows as the duration of post-task rest increases. In other words, during early sleep, DBS may have less of an effect on increasing spindle rates versus later sleep where DBS may be more effective at evoking spindles. If sleep memory consolidation focussing on newly learnt tasks is concentrated around early sleep, then perhaps this is evidence for why DBS did not improve learning performance because its effectiveness in increasing spindles may instead be concentrated on later sleep. This should be confirmed and investigated in future studies with a more adequate sample size though.

Regarding the interpretation of the 1st follow-up experiment's results, results of the 1st pilot do suggest that variations of the high barrier and lower shelf reaching task are more difficult than the traditional reaching task. However, these variations are not readily learnable such that their performance can increase and return to the original task's performance level. Critically, the trendline for performance during the barrier-far-well

variant and -2 mm low-shelf variant exhibited nearly horizontal learning trend lines over 7 days each. Considering this was tested with highly trained rats, based on the variants tested, it seems unlikely that one of these variants would act as more difficult but learnable alternative to the original reaching task. However, not all learning is gradual, but sometimes sudden insight is gained through sleep for example. Through this saltatory learning procedure, it is possible that if the training on the different variants was continued, then a sudden increase in performance could occur. This “eureka” moment could result from the rat learning the necessary change in technique to make the new task substantially easier for example. Therefore, it is not conclusive that these variants of the single-pellet reaching task are not learnable, but it is challenged by the fact that they were not learnable in even highly trained rats over 7 days for example.

Regarding the interpretation of the 2nd follow-up experiment’s results, the conclusion that the rats cannot sense if the pellet is present or not before reaching cannot be confidently made. This is due to a few important observations and nature of the task. First, during this pilot study, it was not uncommon for the rats to claw and bite at the reaching slit and door before the task had begun. Second, after a successful grab of a pellet, sometimes the rats would then make another reach right after eating the first. Lastly, there is the fact that the rats were trained to simply reach whenever they were in the reaching box, particularly when the door was opened. This was accompanied by no punishment for attempts at reaching, only an occasional reward in the form of a successful pellet reach. Therefore, it can be argued that the rats were simply trained to readily attempt many reaches while in this environment without repercussion, and thus were impartial to whether the pellet was physically placed or not with all other factors

being the same. I argue then that this pilot alone cannot conclude whether the pellet was sensed by the rats on the low-shelf or not. However, future work could examine the reaching video for evidence of sniffing behaviour to locate the pellet.

A point regarding the amount and timing of TMR tone delivery: Oudiette and Paller (2013) raise the potential issue of sensory habituation to a TMR stimulus if exposure is too great, limiting promoted reactivation. Currently there is no clear criteria for how much TMR stimulus to deliver for optimal effect, but comparisons can be made with successful rodent studies such as Bendor and Wilson (2012) and Rothschild et al. (2017). Bendor and Wilson delivered each TMR tone about 2.1% of the duration of total rest. This resulted in about 154s of a specific sound being played in 800ms snippets over 2 hours of rest. Rothschild and colleagues delivered each TMR tone about 4% of the duration of total rest (although there were epochs with no TMR tone delivery, so if these are included in total rest time, then only 1.6% of the total rest duration was TMR tones). This resulted in about 66s of a specific sound being played in paired 200ms bursts over a 25-30 minute rest epoch (exact details of tone delivery is quite nuanced, so please refer to Rothschild et al. (2017)'s online methods and figure 3a for more detail). In my experiment, the TMR tone was played about 2.05% of the duration of total post-task rest. This resulted in about 92s of the tone being played in 500ms blocks (every 3-4 seconds during SWS specifically) over a 75 minute post-task rest epoch. Therefore, the percent of total rest duration that the tones played is comparable across all three studies. Also, the length of the TMR sound clip used in this study was 500ms, versus Bendor and Wilson's 800ms and Rothschild and colleagues' 200ms bursts, placing it intermediate in length and thus perhaps not an issue needing adjustment in future work. Lastly, the total duration of

tone delivery in my experiment at 92s was also in-between the 66s and 154s durations of Rothschild et al. (2017) and Bendor and Wilson (2012), respectively. It is also interesting to note that both of the previously mentioned studies used multiple other control tones as well, and perhaps this could be incorporated.

Therefore, the current TMR paradigm in this study does not likely pose a risk of becoming habituated and potentially weakening its influence as a tool for biasing memory replay when compared to other studies. This tone discussion also suggests that perhaps the length and frequency of TMR delivered during sleep may not be a cause for the lack of a learning improvement in this work.

5.2 Limitations

There are a few important limitations I would like to outline that prevent a conclusive closure to the original hypothesis of the effect of TMR and DBS on motor learning in rats. Some of these points will lead to some future directions via improvements that can be made in the design or different questions to approach (see next section).

Firstly, there is the issue of the experimental group's combination of TMR and DBS without more groups testing the effect of just TMR or DBS by themself. Please refer to the Appendix for an explanation of why this combination was chosen and the drawback from an experimental design perspective. Expanding on this point, it is important to realize the possibility of an interaction effect between TMR and DBS both being present. The original crude prediction was that if TMR is able to bias memory reactivation and DBS can increase spindle density, that these two processes could work synergistically to enhance learning and behaviour. However, the mechanisms and timing

of TMR and DBS are not fully understood, leaving the possibility that there may be interference between these two processes.

In this experiment for example, the TMR tone was delivered 200ms after the delivery of the DBS pulse, to ideally align the onset of DBS induced spindles with the approximate onset of the TMR tone, based on work by Eckert et al. (2021). However, Rothschild et al. (2017) found when demonstrating that TMR tone induced auditory cortex activity did predict subsequent hippocampal reactivation (when around SWRs), that this process occurs approximately in a timeframe of 0-150ms from the delivery of the tone. This is based on figure 2C from their work (also in Figure 19, left-side), depicting mean CA1 z-scored peri-SWR time histogram data which spikes around 0-150ms from SWR onset, whilst also acknowledging that this spike was highly correlated with an increase in auditory cortex spiking, and even preceded by it. Figure 3C then demonstrates that the auditory tone almost instantaneously evokes an increase in auditory cortex activity (also in Figure 19, right-side), therefore suggesting that the window of largest induced change in activity occurs between 0 and 150ms from tone delivery. Applying this knowledge to my experiment's protocol, it is possible that with a 200ms delayed TMR tone delivery after DBS pulse delivery, that the resulting induced change in CA1 activity, and therefore potential memory reactivation, could occur during this 0-150ms window after tone delivery. This timing of potential TMR induced memory reactivation may be sub optimally aligned with the window of becoming associated with the peak in DBS induced spindle at around 100-300ms, therefore potentially missing the synergistic effect of applying these two techniques together (Figure 20). See next section for further explanation of the reasoning and proposed solution.

However, the current timing of TMR and DBS stimulation may not be just unoptimized but may also interfere with hippocampal-cortical communication due to potentially biasing a spindle-SWR coupling instead of SWR-spindle coupling. However, currently there is no evidence for this reversal in coupling from Figure 11 depicting no timing preference for spindle onset with respect to SWR onset. There is likely an issue with the results in the SWR-triggered spindle events though (Figure 11), so this is a question to revisit in future analyses.

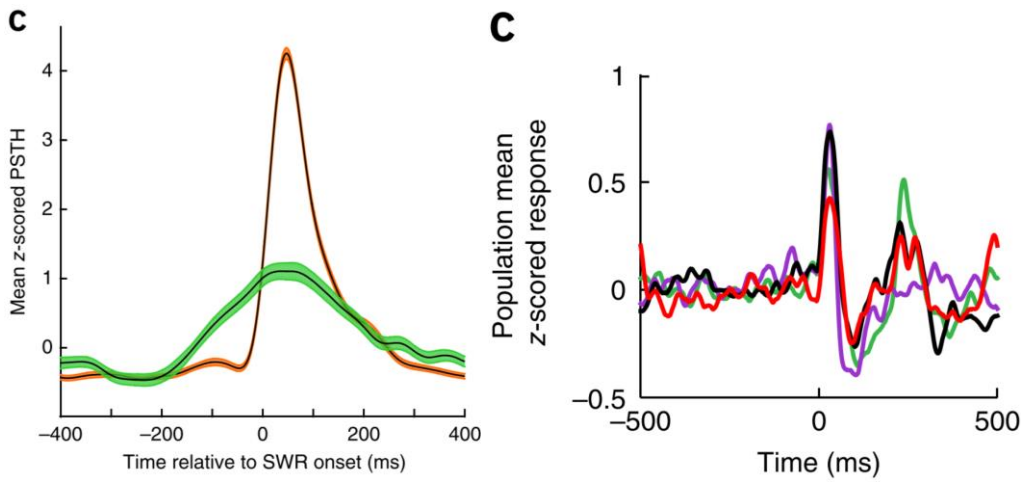

Figure 19. Figures from Rothschild et al. (2017). Left: from figure 2C in original paper. Original description: "Mean z-scored SWR-triggered spiking histogram across all SWR-modulated neurons for AC (green) and CA1 (orange). Shaded area indicates s.e.m.". Right: from figure 3C in original paper. Original description: "Population average of *z*-scored PSTHs of all AC cells that were defined in both the first and last daily sound sleep epochs (*n* = 496 PSTHs from 248 cells) to the different sound stimuli". PSTH – peri-SWR time histogram, AC – auditory cortex.

Second main limitation pertains to the nature of the single-pellet reaching task for comparing learning. Originally the single-pellet reaching task was utilized with a clinical

purpose of testing limb movement during post-stroke recovery in rodents (Klein et al., 2012; Whishaw & Kolb, 2004), and the many papers using this task for stroke, lesion and drug studies is evidence for its utility. However, the use of the single-pellet reaching task for comparing learning rate between healthy rodents based solely on an experimental manipulation like TMR or DBS is limited. This may be in part due to the natural high variability in skill between rats on this task. Indeed, the reaching performance of the 6 rats in this experiment were deemed as typical by Dr. Whishaw, the creator of the single-pellet reaching task (I.Q. Whishaw, personal communication, September 13, 2022). Therefore, statistically it becomes very difficult to find group effects when variance within groups is naturally high. Lesion or stroke induced behaviour changes in limb movement and reaching are likely dramatically more effective in being detected by this task than comparing two healthy groups with an experimental treatment that may only yield a small effect. To review what was mentioned in section 4.1 Experiment Redesign Justification, there was also the issue of power for this task. Using the variance and data from the 6 tested rats, it would require 80 rats for 0.8 power assuming a 20% increase in learning for the experimental group. For an experiment with electrophysiology recordings, I argue that a group size of 80 would not be feasible for many laboratories.

Building on the issue of the nature of the task and concerns of power, there was also the issue of not understanding what aspect of the reaching task was being learnt. It is believed to be natural for rodents to grasp food they encounter in the wild on the ground using their digits, but it is unclear what aspect of the reaching task is novel motor learning and whether this is truly reflected in success/fail scoring. In lesion studies using the reaching task, it was found that rats were able to improve on the task after a lesion not

by relearning the same limb movement, but by making a new compensatory motion to grab the pellets (Whishaw & Kolb, 2004). But for healthy populations, whether this task can demonstrate behavioural differences that are from novel learning and not due to recovery is not as clear.

Related to the pre-training and paw assessment of the rats, there is an issue of varying levels of understanding the purpose and goal of the task during early training. The fact that not all rats consistently did 20 reach attempts during the first few days of training is indicative that some did not understand the nature of the task and therefore lagged behind in performance versus those that did understand. This contrast is seen when comparing rat 1C and rat 2C who had initially poor and initially good performance on the task, respectively. Despite efforts to limit and equalize pre-training experience during paw assessment, not all rats understood the task requirements like others. This resulted in added variance to the results not due to motor learning, but instead due to behaviour such as idling and inattention. This is the reasoning that went into designing a variation of the reaching task that was similar, yet more difficult to the original. If rats were trained on the original version, and then switched to the more difficult variation, then this would ensure that all rats properly had time to understand and achieve their own peak performance in the reaching task, thus levelling knowledge of the task. Unfortunately, a suitable variation was not found, but this concern nonetheless exists for the original experiment and may be another explanation for the high variability in performance observed.

5.3 Future Directions

With limitations in mind, I would like to propose a few changes that address some of them, in addition to a few comments for future work regarding this experiment.

Firstly, regarding the potential issue of TMR and DBS combined into a single group, with potentially interfering effects. An obvious improvement is to include separate groups for DBS only and TMR only to single-out and understand the individual effects of these techniques on the experimental outcomes. If incorporating a combined TMR/DBS group again, it may prove helpful to alter the timing of their delivery. For example, instead of delivering the TMR tone 200ms after the DBS pulse, deliver the tone 50ms after the DBS pulse (Figure 20). This may provide the necessary time for the TMR tone to bias memory reactivation and SWR onset just before or during the onset of the DBS's induced spindle event instead of vice versa. This timing or other timings between DBS and TMR based on other evidence for ideal SWR-spindle interactions could help improve hippocampal-cortical communication. However, this suggestion may not be necessary because the influence of the TMR tone in Rothschild et al. (2017) was found to last even after sound delivery ended. They found that auditory cortex activity was still biased by sounds occurring up to 14 seconds before such that it still had an influence on subsequent CA1 reactivation activity (however, the limit of 14 seconds was due to the timing of when the next sound could be delivered). Also, there could be interference of the two modulation techniques, because Rothschild et al. (2017) also demonstrated that SWR density decreased during sound delivery versus when no sound was being played. Therefore, the timing of delivering TMR before DBS may pose an interfering effect due

to a decrease in SWR density which may implicate a decrease in hippocampal-cortical communication for memory consolidation.

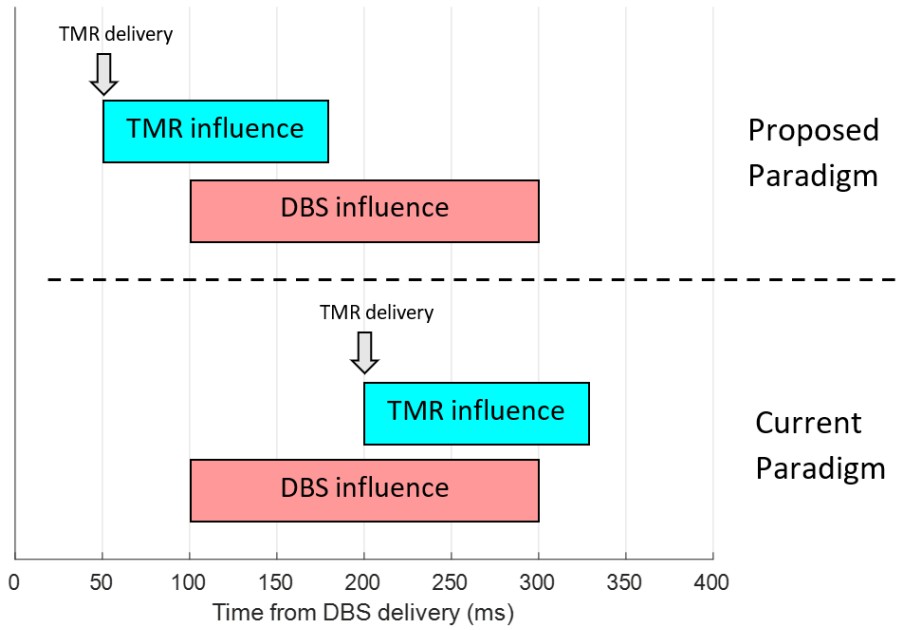

Figure 20. DBS and TMR timing paradigms. Comparison of proposed and current timing paradigms for experimental manipulations. The example proposed paradigm may allow for more optimal SWR-spindle coupling by moving TMR delivery to an earlier time versus the current paradigm. DBS influence box is 300ms wide and TMR influence box is 150ms wide. DBS influence range is based on the findings of Figure 12. TMR influence range is based on the width of the peak in CA1 activity in Figure 19 (left-side).

Regarding the issue of varying levels of understanding the reaching task during early days of training, there are possible adjustments to the pre-training procedure that could improve this. Tang et al. (2018) performed a similar experiment utilizing transcranial magnetic stimulation (TMS) with mice performing the single-pellet reaching task, but had a more robust method for pre-training and accounting for rat's individual

differences before the experiment begun. Tang and colleagues allowed for a range of 1 to 4 days of pre-training for each rat, with pre-training ending once a rat made 20 reach attempts within 20 minutes (considered “baseline level”). Rats that took only 1 day of pre-training were considered “fast-learners” and were balanced across control and experimental groups. The same was done for rats that took 2 to 3 days to reach baseline level as “intermediate learners” and 4 days as “slow learners”. Rats that took greater than 4 days were not included in the experiment. This simple procedure would help reduce the effect of individual differences on finding group effects through better group balancing.

Related to TMR stimulation, one technique that could potentially improve its effectiveness is to deliver TMR tones during cortical slow oscillation up-states. In addition to work by Antony et al. (2018) who found evidence for specificity of the timing of TMR delivery, Ngo and Staresina (2022) similarly demonstrated in a human study that TMR delivery during cortical up-states was effective at reducing forgetting of a word association task versus delivery during slow oscillation down states. Although this thesis work was an attempt to replicate Bendor & Wilson (2012)’s TMR methodology, future work may require more precision of TMR delivery to reliably elicit an effect on learning. However, if cortical up states and spindle activity are driving memory consolidation, then the tight relationship of increased spindles during slow oscillation up states demonstrated in humans after learning may not apply to the same degree in rats which did not demonstrate an increase in spindles necessarily during cortical up-states after learning (Mölle et al., 2009). Therefore, the timing of TMR tone delivery if tuned to cortical up-states may only be advantageous for humans and not necessarily for rodents.

Regarding DBS, there is the possibility that the electrical stimulation delivered interfered with endogenous cortical activity. Studies have supported that stimulation of the cortex with electrical pulses can induce a temporary excitation, and then a relatively prolonged inhibition in cortical regions (Chung & Ferster, 1998; Logothetis et al., 2010). However, behaviourally the resulting performance on the single pellet reaching task for the experimental group was found to be statistically no different from the control group, suggesting also that they did not perform worse than controls. Nonetheless, considering potentially unaccounted for side-effects of direct cortical electrical stimulation via DBS, it may be worth exploring stimulating other regions and thus potentially not disturbing motor cortex activity as severely. Eckert et al. (2021) found in their DBS protocol (which was used here) that the resulting change in evoked spindle activity was seen in both hemisphere's motor cortex electrodes, regardless of which side the stimulating electrode in motor cortex was implanted. This suggests that the current DBS protocol has far ranging effects on the cortex. Previous work has examined the effects of stimulating the thalamus directly though, which is thought to be the origin of thalamocortical spindle events (De Gennaro & Ferrara, 2003). A notable work by Latchoumane et al. (2017) used optogenetic stimulation of the thalamic reticular nucleus specifically to induce spindle events and resulted in improved contextual fear memory. Comparing the current DBS paradigm and Latchoumane et al. (2017), a difference aside from use of optogenetics for stimulation was that the resulting spindle entrainment in Latchoumane et al. (2017) which did not result in a net increase of spindles, but rather increased slow oscillation-spindle coupling. Eckert et al. (2021) reported both a net increase in spindles and increased slow-oscillation-spindle coupling. This suggests that these methods may differ, however it may

be worth exploring the stimulation paradigm of Latchoumane et al. (2017) on the learning of a motor task.

In terms of analysis, there are still some questions that can be investigated with the LFP and video data collected. For example, periods of motionlessness during rest epochs that did not qualify as REM sleep or NREM were considered “quiet wakefulness” (QW). Quiet wakefulness is not formally a stage of sleep; however, it shares some properties with sleep and could be interpreted as an intermediate state between active wakefulness (which necessitates movement) and sleep (seen as NREM sleep or SWS in this experiment). QW entails a lack of movement and exploration, but REM and SWS detection algorithms do not consider these periods as meeting the threshold of their respective phases (see section 2.7.3 Electrophysiology). Similar to NREM sleep having the cortex in a synchronized state, cortical neuron populations also behave in a more coordinated manner during QW, fluctuating between up and down states (Poulet & Crochet, 2019; Steriade et al., 1993). As described by Harris and Thiele (2011), “desynchronized” and “synchronized” states exist on a continuum, so it may be interesting to include this period in future sleep analysis. For the video data, work is currently being conducted to analyze the reaching trajectories in hopes of finding evidence for measurable differences during learning that may not be caught by the coarse “success/fail” scoring traditionally used for the reaching task.

Moving on from limitations and analysis, there are a few points that can be considered if future work will still employ the single-pellet reaching task. Other studies utilizing the same task can be used as inspiration for aspects that reflect better methodology. This includes the use of a raised pedestal pole to hold the pellet for

reaching instead of a shelf (Zemmar et al., 2015). This design has the advantage of preventing simple dragging motions to obtain the pellet, but instead forces the rat to grasp the pellet. The enforcement of a proper grasp is to engage fine-motor skills to be learnt versus a cruder dragging/swiping motion. This was the intended role of the barrier used in this experiment, but some rats still exhibited occasional dragging-like behaviours during reaching by sliding the pellet up the barrier. Considering the time-intensive nature of manually training each rat every day to perform the task, automated versions of this task have been demonstrated to dramatically speed up this process, such as the open-source and low-cost design by Wong et al. (2015). Such automation could even allow for more rats to be tested in a cohort, readily allowing for larger group designs and helping tackle the issue of low power mentioned in the previous section.

There is the important consideration of using a different task to assess motor learning in rats. The single-pellet reaching task as mentioned before may not be a sensitive enough test designed to measure differences in learning between two healthy groups. However, it is not readily clear what task could alleviate this same issue. The complexity and expectations of the task are obviously limited by nature of being performed by rats. There are many motor task studies available in the human literature, and studies testing TMR and TES on its learning have been mentioned already. DBS research in humans is quite limited and difficult, however other questions pertaining to TMR and motor learning can certainly be tested with human studies. This experiment's hypothesis of combining DBS and TMR may not be readily testable in a human setting, but questions pertaining to TMR and the resulting memory processes could be tested. For example, Abdellahi et al. (2023) tested motor sequence learning in humans and claimed

to demonstrate reactivation was detected based on a TMR tone delivered during sleep via recorded EEG activity. In order to detect this reactivation, they trained a linear discriminant analysis classifier on 80ms snippets of time-domain amplitude averages from EEG data during waking behaviour, and then applied the classifier to sleep data through a sliding window.

Although the method of memory reactivation detection used by Abdellahi et al. (2023) was crude compared to using unit activity, such methods of studying memory reactivation may be a useful tool for asking more questions about the efficacy and mechanism of TMR on motor learning in humans that goes beyond just behavioural results.

6. Conclusion

It has been shown that sleep and memory processes are longstanding topics within neuroscientific research. Despite their importance to our daily functioning, the purposes and mechanism behind some of these processes are not well understood. How motor memories operate and be enhanced were the focus of this thesis work. The two techniques incorporated were TMR and DBS for potentially enhancing motor memory consolidation. TMR has shown success in human studies but there is limited evidence for its efficacy in rodents for motor memory tasks. DBS has not been applied to motor memory task studies, but Eckert et al. (2021) developed a protocol for reliably inducing spindles, which may enhance memory consolidation and learning. Previous work in the Tatsuno laboratory made attempts at testing TMR's effectiveness in improving single-pellet reaching performance but suffered from some design issues and failed to find an effect. This work set out to improve the design of previous tests and to test the hypothesis that the combination of both TMR and DBS in experimental rats would increase the rat and/or peak performance in a single-pellet reaching task versus a control group receiving neither manipulation.

To test this hypothesis, it was originally planned to have 24 male rats split evenly into an experimental and control group. However, 6 rats (4 experimental, 2 control) were only tested due to interim results and discovery of further design problem. With these 6 rats, all were implanted with LFP electrodes and tested for 14 days plus a one week later probe trial on the single-pellet reaching-task. Each day entailed a before-task rest epoch, task epoch and a post-task rest epoch. For experimental rats only, TMR was delivered during training before the door opened before each reaching trial. TMR and DBS was

then delivered during SWS for experimental rats in post-task rest, but only TMR was delivered for control rats. After recording, electrophysiology data was classified into sleep stages and searched for spindle and SWR events.

Reaching success results showed that there was no statistically significant difference between groups. Spindle detection found a statistically significant increase in amount of spindle events in post-task rest for experimental rats versus control rats, likely due to the DBS protocol. Peri-event histogram results showed that spindle onset typically increased in occurrence 100-200ms after DBS pulse events as well, supporting results found by Eckert et al. (2021). SWR rates did not statistically differ between groups.

To address some of the issues of the original experiment and prepare for a new proposed experiment, a few pilot studies were conducted. It was found that none of the tested single-pellet reaching tasks was suitable as a more difficult but learnable alternative. Testing whether the rat can sense or see a pellet on a lowered shelf was inconclusive as well. Lastly, a power analysis suggested that to achieve .8 power would require 80 total rats, which is currently an infeasible size for the current experiment design.

This work was concluded with a few suggestions for improving the design of the experiment and the critical recommendation of not using the single-pellet reaching task for comparing learning performance, largely due to its inherent low power to find effects unless group sizes are very large. Due to these issues and others, it is inconclusive whether TMR and DBS can enhance motor memory learning and performance on the single-pellet reaching task. However, an improved and expanded experiment design may

prove fruitful because of supporting DBS and TMR literature in addition to the confirmed presence of an increased spindle rate due to DBS in this study.

Appendix

Two important notes regarding the purpose and questions of this experiment. First, originally there was a 2nd research question, namely: is there effect of sex on the effectiveness of DBS and TMR on the rate of motor learning or overall performance? Pilot work I conducted in preparation for this experiment included female rats in order to test this research question. However, it was determined that inclusion of female rats posed a behavioural confound. It was observed and determined that female rats are more severely affected by food restriction than male rats, resulting in more desperate and excited reaching behaviour during training when under the same percent body weight food restriction as males. This issue is compounded by that fact that inclusion of the female sect requires enough rats to compose an adequately sized group to have sufficient power to find an effect if it exists. However, from a practical standpoint, it was difficult to plan for more than 24 rats total, which would have only resulted in six female rats each if splitting control and experimental groups evenly, and then splitting evenly by sex. Therefore, for the resulting experiment it was decided to test only male rats to alleviate these issues, and then in the future to include females if results showed promise for their inclusion.

Secondly, regarding the combination of DBS and TMR into a single experimental group. From an experimental design standpoint, it would be ideal to have four groups in total: control, TMR only, DBS only, or TMR and DBS. With this design, it could be fully tested whether any effects or learning differed as a function of TMR or DBS by themselves, in addition to testing whether a combination is effective or necessary. However, due to practical limitations for a Master's thesis project, the experiment would

have doubled in planned work and time by including a TMR only and DBS only group. Therefore, in order to maximize the chance of finding an effect and supporting the hypothesis with the time available, TMR and DBS were combined to become the experimental group. If an effect was found, then a follow-up study could be done to test whether TMR and/or DBS was responsible for the effect.

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