UNSUPERVISED DETECTION OF CELL ENSEMBLES IN RATS' PRIMARY MOTOR CORTEX DURING ONLINE AND OFFLINE PROCESSING

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DEDICATION

To my love, my son and my dear parents for their endless support and encouragement.

ABSTRACT

Motor actions engage intricate neural processes, spanning active learning phases and crucial offline periods, notably during sleep. Online learning involves diverse neural dynamics, while sleep is known for its role in skill consolidation. While numerous studies have contributed to our understanding of information processing during online and offline learning periods, these investigations have often focused on specific learning phases, leaving the intricate relationships between diverse online learning neural activities and sleep processing relatively unexplored. Here, we embarked on a comprehensive analysis aimed at unraveling the interplay between primary motor cortex (M1) neural activity during reach-to-grasp skill learning and sleep, employing an unsupervised framework. During online training, our findings uncovered four neural dynamics related to the motor execution, with compelling evidence of their replay during post-training sleep, both in Rapid Eye Movement and Slow-Wave Sleep (SWS). Moreover, our data revealed that all cell ensembles, irrespective of their dynamics during the task, exhibited substantial reactivation during spindles coupled with slow-oscillations in SWS. Further exploration on the cortico-hippocampal communication led us to investigate the activation patterns of M1 cell ensembles during hippocampal sharp-wave ripples. Our results demonstrated the dynamic suppression and enhancement modulation of M1 cell ensembles during SWS-ripples across learning days suggesting complex cortico-hippocampal dialogues associated with sensorimotor learning task. We thus contributed to understand the extensive details of neural mechanisms underlying motor learning tasks during online and offline processing periods.

PREFACE

The experimental design and data recording was performed by Dr. Michael Eckert (Eckert et al., 2020).

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LIST OF ABBREVIATIONS

CAD	Cell Assembly Detection
EMG	ElectroMyoGraph
ICA	Independent Component Analysis
LFP	Local Field Potential
PCA	Principal Component Analysis
PC	Principal Component
PETH	Peri-Event Time Histogram
REM	Rapid Eye Movement
sd	Standard Deviation
sem	Standard Error of Mean
SO	Slow-Oscillation
SWR	Sharp-Wave Ripple
SWS	Slow-Wave Sleep

1. Introduction

This section represents a brief overview of terminologies, concepts, theories, and recent findings related to this research study.

1.1. Neural information encoding

The fundamental units of brain network, neurons, are central to the process of information encoding. Two primarily theories have been proposed to explain how neural circuits encode information: single-cell encoding and multiple-cell encoding or cell assembly (Hebb, 1949; Sakurai, 1996).

The single-neuron encoding theory focuses on role of individual neurons in encoding and processing information. According to this theory, the activity of a single neuron can carry meaningful information. Each neuron's firing rate or pattern can represent specific sensory inputs, motor outputs, or cognitive processes (Barlow, 1972).

The cell assembly theory originally proposed by Donald Hebb posits that information is encoded and processed through the coordinated activity of multiple interconnected neurons acting together as a functional unit. Hebb suggested that when two connected neurons are repeatedly activated together, their synaptic connections are strengthened, leading to the formation of cell assembly representing a specific meaningful sensory or cognitive information. Cell assemblies are believed to be the fundamental units of information processing in the brain, with their precise timing and pattern of activation play a crucial role in information representation (Hebb, 1949).

While the single-neuron activity has been a convenient focus for many experimental studies, its inherent variability, limited signal-to-noise ratio, and challenges in preserving individuality make it insufficient as the sole basis for information processing in the brain (Sakurai, 1996). Thus, understanding how neurons collaborate as cell assemblies and neural circuits appears to be crucial for unraveling the complexities of information processing in the brain. Cell assemblies have been implicated in various brain functions, including memory formation, learning, perception, and decision-making (Wickelgren, 1992; Sakurai, 1996; Harris et al., 2003; Buzsáki, 2010).

Although the original Hebbian's assembly hypothesis required anatomical connection as a perquisite for the formation of cell assembly, many studies modified this definition by relaxing the anatomical constraint and considering groups of either anatomically or functionally connected neurons that cofire to represent a distinct cognitive entity or percept. Neural clusters under the umbrella of the modified definition sometimes are called as cell ensembles (Churchland and Sejnowski, 1992; Miller, 1996; Harris et al., 2003; Buzsáki, 2010; Russo and Durstewitz, 2017).

1.2. Motor learning

Motor learning is a dynamic process that involves enhancing or refining motor actions through practice and repetition (Tian and Chen, 2021). Generally, it is explored from two core aspects: motor sequence learning, encompasses incremental acquisition of sequential movements to form a well-articulated behavior, and motor adaptation which examines our capacity to adjust and compensate for environmental

changes (Doyon et al., 2009).

The process of motor learning unfolds through several stages. It begins with the initial acquisition phase, marked by rapid performance improvements. Subsequently, the consolidation phase ensues, characterized by the gradual automation of acquired skills. Finally, a long-lasting memory is formed to maintain the acquired skills over time (Karni et al., 1998; Hikosaka et al., 2002; Kargo and Nitz, 2004; Doyon et al., 2009).

Extensive research in humans and animals, including rodents and primates, has demonstrated the remarkable persistence of motor skills over varying time spans. These retention periods can range from mere hours to days, and in some cases, extend across decades, with little interference from concurrent skill acquisition or performance (Shadmehr and Holcomb, 1997; Hikosaka et al., 2002; Walker et al., 2003; Buitrago et al., 2004; Matsuzaka et al., 2007; Park et al., 2013; Kawai et al., 2015).

The process of motor learning involves several key brain areas that work together to acquire and refine motor skills. This network includes the cerebellum, which is pivotal for coordinating and refining movements, and the basal ganglia which is responsible for selecting and initiating motor actions. The primary motor cortex (M1)- as a core center of motor learning which receives projections from thalamus subregions and various cortical regions (Muñoz-Castañeda et al., 2021)- executes and regulates voluntary movements, while the supplementary motor area and premotor cortex (or secondary motor cortex in rodents) are instrumental in planning and organizing movements (Tian and Chen, 2021). The prefrontal cortex

contributes to decision-making and strategic planning in motor learning (Lee et al., 2020). Spinal cord transmits motor signals and coordinates precise movement execution (Vahdat et al., 2015). The involvement of the hippocampus in motor learning tasks extends to acquiring arbitrary sensory-motor connections, specifically in the acquisition of arbitrary visuomotor mappings (Wise and Murray, 1999). In the other word, Hippocampus might not have a direct role in generating or timing motor responses during learning tasks. Instead, it seems to indirectly contribute by possibly creating a temporary behavioral state needed for motor learning to happen (O'Keefe, 1999).

Recent findings demonstrated that when individuals learn a motor task, it leads to the creation and preservation of new synapses, which are indicative of long-lasting memories being formed. This process involves alternations in neuronal excitability and synaptic plasticity within key motor regions in the motor cortex (Xu et al., 2009; Peters et al., 2014; Kida et al., 2016; Qiao et al., 2022).

Long-term recordings in rodents showed that during motor learning, individual neurons in the motor cortex do not reliably predict trial success. However, analyzing the activity of the entire neuronal population together, especially with fine-scale resolution, improved predictions of success. This collective activity across neuronal populations is vital for motor learning and augments the ability to predict successful motor tasks (Laubach et al., 2000). Furthermore, two-photon calcium imaging of the neural population in the mice's motor cortex revealed increased synchronized activity among functionally related neurons during motor behavioral training as the mice learned, suggesting the emergence of correlated cell ensembles that might underlie

learning-related changes in the cortical circuitry (Komiyama et al., 2010).

1.3. Neural activity dynamics associated with motor learning

Motor actions are accomplished by complex and varied changes of neural activity patterns to compensate multiple environmental noise factors and provide the most optimal action (Rokni et al., 2007). Calcium imaging data represented different neural population dynamics including both enhancement and inhibition activity in the rats' motor cortex layer 2/3 during level-press task with suppression modulation as the prevalent type (Peters et al., 2017). The inhibition modulation of neural ensembles has been suggested as a mechanism for upholding a steady framework for motor activities in the zebrafish brain (Liberti III et al., 2016). The analysis of neural populations in the monkey's primary cortex revealed differences in the fundamental structure of neural activities underlying hand control during grasping and arm control during reaching, suggesting specialized neural circuits mechanisms governing different types of motor activities (Suresh et al., 2020). Furthermore, a research study on monkey unveiled separate neural spaces in motor cortex for preparing and executing movements, diverging from expected connections. Despite this separation, activity in the preparation area consistently influenced subsequent movement-related actions, highlighting a distinctive population-level strategy where shared neurons conduct separate but linked computations for movement planning and execution (Elsayed et al., 2016).

1.4. Sleep function in memory and learning

Sleep is widely recognized for its pivotal role in memory and learning processing (Diekelmann and Born, 2010). It serves as a primarily facilitator of memory consolidation, while memory encoding and retrieval are most efficient during wakefulness (Walker and Stickgold, 2006). Consolidation is the intricate process by which newly formed, initially unstable memories are transformed into more stable and semantic representations and ultimately integrating into the existing long-term memory network (Müller and Pilzecker, 1900). This process involves the active re-processing of recent memories within the same neuronal networks initially engaged in their initial encoding. Theoretical models suggested that sleep, characterized by offline periods, appears to be the most conducive environment for effective consolidation, reducing interference between encoding and consolidation processes and minimizing potential disruptions or cognitive disturbances (McClelland et al., 1995). Multiples theories have proposed to explain mechanisms underlying memory consolidation, especially declarative memories in the brain. The following are some of the prominent theories:

- Synaptic Consolidation Theory: According to this theory, memory consolidation is mediated by strengthening of synaptic connections between neurons. As memories are repeatedly retrieved and reactivated, synaptic connections become more robust, leading to long-term memory storage. This phenomenon often associated with late-stage long-term potentiation, takes place at the synaptic and neural circuits shortly after the learning process. (Hebb, 1949; Dudai, 2004; Diekelmann and Born, 2010).

- Systems Consolidation Theory: This theory suggests that memory consolidation involves the gradual reorganization of neural circuits across different brain regions. In declarative memories, initially, labile and susceptible-to-disruption information rely on the hippocampus. However, over time, they become integrated into stable cortical networks, capable of long-term information storage. The consolidation process, as suggested by this theory, unfolds in two stages (Buzsáki, 1989; McClelland et al., 1995; Tulving, 2002; Diekelmann and Born, 2010). It should be noted that systems consolidation theory and its related models primarily emphasize on the declarative memory representation.
- Multiple Trace Theory: It proposes that even after consolidation, memories continue to rely on the hippocampus, and multiple traces of memory are stored in both the hippocampus and the neocortex. The hippocampus is involved in the retrieval of episodic details, while the neocortex holds the semantic aspects of explicit memory (Nadel et al., 2000).
- Sleep-Dependent Consolidation Theory: Emphasizing the role of sleep in declarative memory consolidation, this theory suggests that during sleep, which serves as an unencumbered period for information encoding, memories are replayed or reactivated. This process aids the transition from short-term memory to more stable, long-term memory (Marr, 1970; Wilson and McNaughton, 1994; Skaggs and McNaughton, 1996; Nádasdy et al., 1999; Sutherland and McNaughton, 2000; Diekelmann and Born, 2010).

In summary, while these theories are not mutually exclusive, they provide

different perspectives on the complex process of memory consolidation. Synaptic Consolidation Theory emphasizes the role of synaptic changes, Systems Consolidation Theory explores the shift from hippocampal to cortical control, Multiple Trace Theory suggests continued hippocampal involvement, and Sleep-Dependent Consolidation Theory highlights the importance of sleep in this process.

1.5. Memory reactivation/ replay

Memory reactivation or replay involves the strengthening of past experiences during offline periods following online encoding (Wilson and McNaughton, 1994). During the replay, neural ensembles exhibit sequential reactivation, reflecting predefined patterns of neural activity associated with specific behavioral sequences or measurable similarities (Skaggs and McNaughton, 1996; Nádasdy et al., 1999; Louie and Wilson, 2001; Euston et al., 2007). On the other hand, reactivation is a broader term signifying to activity pattern similarity without a specific temporal relationship and is often measured using zero-lag correlation (Wilson and McNaughton, 1994; Kudrimoti et al., 1999; Peyrache et al., 2009). Moreover, replay analyses concentrate on identifying the correlation between sequential activation of neural patterns with content-specific behavioral information(e.g. spatial/time coding, Bayesian path decoding), whereas reactivation analyses do not prioritize content-specific associations (Tingley and Peyrache, 2020).

Various computational methods and concepts for detecting memory reactivation or replay events have been proposed in related studies, each with its own advantages and limitations. In the following section, we will review some of the most frequently

employed approaches in previous studies.

1.6. Computational methods of replay/ reactivation detection (supervised method)

1. Pairwise correlation: Enhanced activity of hippocampal neurons after a novel experience task has been hypothesized as a potential mechanism for episodic memory processing (Pavlides and Winson, 1989). In order to investigate the evidence of memory reactivation during sleep and how it relates to prior and subsequent wakeful experiences, pairwise correlation was introduced which is one of primarily methods of reactivation analysis in large recorded neural data (Wilson and McNaughton, 1994). Statistically, Pearson correlation measures the strength and direction of the relationship between two variables. In the context of reactivation analysis, the variables of interest were the neural firing rates during different states: waking, pre-sleep, and post-sleep. The procedure involves calculating the pairwise correlations between the firing rates of neurons during waking and comparing these correlations with those during pre-sleep and post-sleep periods. Specifically, the analysis aims to determine whether the correlation between neural firing rates during waking and post-sleep was significantly different than that between waking and pre-sleep (Wilson and McNaughton, 1994). Using this method, it has also been indicated that the temporal order of neural ensemble firing during sleep closely resembled to the behavioral state observed during wakefulness, suggesting the replay of memory during sleep (Skaggs and McNaughton, 1996). However, it faces challenges in establishing a

clear correlation between prior experience and their impact on the firing rate correlation of post-sleep neural activity with that observed during wakefulness. Explained Variance (EV) method has been advanced to take into account the pre-existing structure of the data and assess how much additional variance in the post-sleep state can be explained by relative values in the wake state while considering the effects of pre-sleep experiences (Qin et al., 1997; Kudrimoti et al., 1999; Hoffman and McNaughton, 2002). Mathematically, EV is calculated as follows:

$$Explained\ Variance\ (EV) = r^2_{w,post|pre} = \left(\frac{r_{W,Post} - r_{W,Pre} r_{Pre,Post}}{\sqrt{(1 - r^2_{W,PRE})(1 - r^2_{PRE,POST})}} \right)^2$$

where $r_{x,y}$ is the correlation between epochs x and y for a given cell pair. Traditional pairwise correlation-based methods suffer from noise correlation sensitivity, temporal reactivation constraints, and overlooking neural spatiotemporal correlation (Chen and Wilson, 2017; Tingley and Peyrache, 2020).

2. Template Matching: In this method, peri-event time histogram (PETH) is defined during a user-defined window locked around a specific time point as an average pattern of neural activity during behavioral trials. This template is then correlated with the neural spike count matrix during sleep to measure reactivation strength in terms of correlation values. The template neural activity matrix may be compressed or expanded at different scales to identify similar neural activity during sleep with different speeds (Nádasdy et al., 1999; Louie and Wilson, 2001; Tatsuno et al., 2006; Euston et al., 2007). Although template-matching is able to find the spatiotemporal neural patterns, being dependent on the template which is

created by a user-defined temporal scale and user defined temporal resolution as well sensitivity to noise correlation and outliers are some of limitations remained in this method (Chen and Wilson, 2017).

3. Subspace analysis: It involves representing neural data using dimensionality reduction techniques to map high-dimensional neural space into lower-dimensional space. Principle Component Analysis (PCA) as one of preliminary proposed methods identifies the axes in the original data that maximize variance. Each principal component (PC) corresponds to an eigenvector of the neural activity correlation matrix, representing the direction of the most captured variance. PCA can be applied to the z-transformed spike count matrix derived from either averaged firing rates across training trials or concatenated trials. Trial window length is selected by the user (Williams et al., 2018). The determination of the number of significant components is based on eigenvalues surpassing the theoretical upper limit of the eigenvalue spectrum, known as the Marchenko-Pastur bound (Peyrache et al., 2009). However, this approach has faced challenges, primarily due to the potential for false ensemble detection under conditions of data non-stationarity (Russo and Durstewitz, 2017). Sleep reactivation analysis is performed by projecting PC eigenvector (for example, PC1) to z-scored neural firing rate data at each time point during sleep. Significant reactivation times are typically defined as instances when the reactivation strength exceeds a specific threshold within the distribution of reactivation values, distinguishing them from background activity (Peyrache et al., 2009; Peyrache et al., 2010). Independent Component Analysis (ICA) is an extension

of PCA, which employs higher-order moment statistics to extract statistically independent components from non-Gaussian signals was utilized in reactivation analysis instead of PCA to overcome on PCA orthogonality assumptions (Lopes-dos-Santos et al., 2013; Van de Ven et al., 2016; Oberto et al., 2022). Estimating the number of ICs is a necessary step and can be performed using the same technique suggested in PCA (Lopes-dos-Santos et al., 2013). The drawbacks of PCA and ICA can be summarized to the linearity, orthogonality (in PCA) which are not necessarily true assumptions in the real brain as well as non-zero-lag correlation detection inability (Russo and Durstewitz, 2017; Williams et al., 2018; Tingley and Peyrache, 2020). Furthermore, reactivation strength within this framework, is simply the sum of the squared firing rates of all neurons at time t and as a result, it's proportional to the quadratic power the temporal firing rate of individual neurons. Thus, this quadratic dependence on firing rate means that reactivation strength is highly sensitive to outliers in the firing rate data. Neurons with very high firing rates can disproportionately contribute to the reactivation strength, potentially leading to false interpretations of memory reactivation based solely on firing rate variations. Therefore, applying subspace methods like PCA or ICA on brain regions with dynamical firing rate or during sleep stages with high level of firing rate non-stationarity like REM could provide potential misleading information. In addition, PCA-derived reactivation strength as a measure of memory reactivation does not directly capture content of memory (Liu et al., 2018). Furthermore, traditional PCA and ICA ignore sequential information in the data, meaning potential loss of important information across data.

Nonlinear dimensionality reduction techniques like GPFA[1] (Yu et al., 2008), dPCA[2] (Kobak et al., 2016) and TCA[3] (Williams et al., 2018) also have been introduced for capturing important neural responses associated with the complex behaviors such as cortical behaviors however such manifold learning approaches have not been used so much in sleep associated with the memory studies.

4. Rank-order analysis: It compares two sequences based on neuronal firing patterns, such as peak firing or the timing of the first spike. Simply counting consistent neurons in the rank order may overlook missing neurons. Instead, the analysis converts firing orders into words and compares the match probability between two words from different states, and then determines the statistical significance of the match. The probability of a repeated sequence for a smaller subset of neurons can be assessed using the set probability, and the chance of a repeated sequence for a longer sequence length is very low. Sequence or rank-order analysis assumes that the firing order contains the main encoded information. Although this method has been used in sleep reactivation analysis (Lee and Wilson, 2002), an important limitation of the rank order method is the uncertainty caused by areas like cortex that have neurons with multiple place fields (Tingley and Peyrache, 2020).
5. Population decoding analysis: It allows to understand how the activity of a population of neurons represents specific information. In this context, "population" refers to a group of neurons that are active simultaneously and are

[1] Gaussian Process Factor Analysis
[2] Demixed Principal Component Analysis
[3] Tensor Decomposition Analysis

thought to be involved in representing a particular feature, such as a location in space or a specific stimulus. The analysis starts by constructing an encoding model (mostly based on the Gaussian place cells tunning curve) for describing how the activity of individual neurons within the population relates to the information being represented. By comparing the observed activity with the encoding model and using methods like likelihood or Bayesian inference, researchers can estimate the most likely representation or feature being represented by the detected population of neurons in another time period. For example, if the population of neurons is involved in encoding the spatial location of an animal, population decoding analysis can be used to determine the most probable location based on the observed firing patterns of the neurons (Zhang et al., 1998; Davidson et al., 2009).

6. Multivariate Pattern Analysis (MVPA), Representational Similarity Analysis (RSA): These methods which are generalized forms of template-matching have been mostly used in human studies using fMRI[1], EEG[2] or MEG[3] data to detect memory replay in human brain (Chen and Wilson, 2023). MVPA techniques utilize machine learning algorithms to train models based on the extracted patterns. These models are trained to classify or predict patterns associated with specific memories or experiences. Once the models are trained, they can be applied to new data to decode or identify instances of memory replay. During rest or sleep periods, the models can analyze neural activity patterns and determine if

[1] Functional Magnetic Resonance Imaging
[2] ElectroEncephalogram
[3] MagnetoEncephalogram

they match patterns observed during the initial memory encoding phase (Deuker et al., 2013; Schönauer et al., 2017). While MVPA uses multiple input resources for training the model, a study used different time-, frequency, and wavelet-domain features of EEG signal to train a classification model with the aim of detecting memory reactivation (Belal et al., 2018). RSA in memory replay, on the other hand, is concerned with comparing and quantifying the similarity or dissimilarity between neural representations associated with different memories during replay. It aims to understand the organization and structure of memory representations by examining the similarity relationships between neural patterns. RSA involves constructing similarity matrices based on pairwise comparisons of neural representations in human data and then conducting statistical analyses to uncover underlying structures or dimensions within the data. It looks like the template-matching method, which evaluates the resemblance in neural patterns between two conditions and determines this similarity according to a predetermined criterion (Diedrichsen and Kriegeskorte, 2017; Chen and Wilson, 2023).

The above methods aim to identify specific neural ensemble activity patterns during a task and then assess the degree of similarity between these patterns and neural activity observed during sleep. Most of these methods are categorized under supervised learning, relying on predefined templates of neural activity from the behavioral state. In other words, replay or reactivation analysis in supervised learning methods relies on the initially defined window length for analyzing the behavioral state. This dependence might introduce bias in the analysis or necessitate further

validations for replay/reactivation detection. Recent advancements in identifying significant ensembles through unsupervised methods could alleviate this dependency on predefined window lengths by analyzing neural data, identifying significant ensembles, and their activation times during recording sessions. This framework may be useful to detect memory replay/ reactivation within unbiased approach by initial detection of ensembles and then looking to their activation times during behavioral and sleep states. Therefore, unsupervised methods might offer deeper insights into neural activity linked to memory processing as they can potentially analyze the entire neural dataset. In the following sections, I will provide a brief overview of some unsupervised methods for the detection of neural ensembles in large-scale parallel neural recordings, illustrating their potential benefits and drawbacks for memory research.

Table 1. Summary of supervised methods of reactivation/ replay analysis

Name	Computation	pros	Cons
Pairwise correlation	Pearson correlation	Simplicity, comparing pre and post activation similarities with waking	Not account for variation between pre and post associated with behavior, Spatio-temporal correlation, sensitive to firing rate outliers
EV	Pearson correlation	account for reactivation changes before and after experience	spatiotemporal correlation, sensitive to firing rate outliers
Template Matching	Cross-correlation	spatiotemporal correlation, replay analysis	User-defined temporal window and temporal resolution (biased analysis), sensitive to firing rate outliers
Subspace Analysis (PCA, ICA)	Dimensionality reduction	Bi-directional reactivation analysis, capturing most neural activity variations	sensitive to neural firing local non-stationarities false reactivation discovery, content-free reactivation, ignore sequential information, linearity and orthogonality (PCA) assumption
Rank Order Analysis	Sequence matching	replay analysis	complexity, applicable to regions with specific neural properties
Population decoding analysis	Bayesian modeling	replay analysis, robust to non-stationarities,	complexity, behavioral bias
MVPA	Machine learning models	brain decoding, flexibility	mainly for human data, complexity, time-consuming for long-term data
RSA	Correlation	Flexibility, insight into cognitive process, data-driven	mainly for human data, require large data, complexity, complex interpretability

1.7. Unsupervised methods of cell ensemble detection

The most important feature of unsupervised methods is their ability to detect cell ensembles during an entire recording and without requiring a user-defined time window which resulted in unbiased analysis of neural data for memory analysis. Here are some of highlighted methods in this category:

1. Unsupervised population decoding: this approach utilizes Hidden Markov Models (HMM) to analyze sequential patterns of neural activity during memory replay or reactivation. HMM is a statistical modeling technique that assumes the underlying process generating the observed data (in this case, neural activity) is a Markov process with hidden states. HMM for memory replay involves the following steps: 1) collecting neural activity data during memory tasks or stimuli presentation, 2) preprocessing the data by extracting relevant features, 3) defining states in the HMM to represent memories or cognitive states, 4) train the HMM using algorithms to estimate transition probabilities- the likelihood of moving from one state to another in the hidden states- and emission probabilities- the likelihood of observing specific outputs based on the hidden states- 5) decoding memory replay instances by estimating the most likely sequence of hidden states, and 6) analyzing the decoded sequences to understand temporal dynamics, transitions, and other replay characteristics (Chen et al., 2012; Maboudi et al., 2018; Tu et al., 2020). Another approach is using latent dynamic models like Kalman filter to decode the desired behavioral parameter outputs from the neural data and then perform the cross-correlation between that decoded output and the predefined template of behavioral information (Rubin et al., 2022).
2. SPOTDisClust[1]: It detects ensembles by constructing an epoch-to-epoch dissimilarity matrix, referred to as SPOTDis, using principles from the optimal transport theory. Indeed, it computes dissimilarity between neural patterns from various epochs by determining the minimum cost of transforming their respective

[1] Spike Pattern Transport Dissimilarity Clustering

cross-correlation matrices into each other, which is analogous to calculating the Earth Mover's Distance for all pairs of neurons and behavioral epochs. This dissimilarity matrix serves as the foundation for density-based clustering, effectively grouping together similar neural patterns. SPOTDisClust has demonstrated its effectiveness in identifying neural ensemble patterns in macaque's V1 cortex associated with the direction of visual stimulus task, making it a promising tool for uncovering complex neural patterns (Grossberger et al., 2018).

3. Similarity-Based Clustering: This method involves preprocessing the neural population activity to extract spike trains from individual neurons, segmenting the spike trains into sequences of fixed length, computing a similarity score between each pair of sequences using a defined edit distance metric - measures the similarity between two strings by calculating the minimum number of operations required to transform one string into the other -, clustering the sequences based on their similarity scores using advanced density-based clustering algorithm called OPTICS[1], and analyzing the resulting clusters to identify cell assemblies. This method has been found to be effective in detecting cell assemblies in both synthetic and hippocampal CA1 neural population activity data and has potential applications in understanding the neural basis of behavior and cognition (Watanabe et al., 2019).
4. SPADE[2]: This method has been designed to detect synchronous spike patterns in multi-channel spike train data. It consists of three steps: a) data mining using

[1] Ordering Points To Identify the Clustering Structure
[2] Spike Patterns Detection and Evaluation

Frequent Itemset Mining[1] to extract potential pattern candidates, b) power spectrum filtering for statistical testing to assess the significance of frequent spike patterns using pattern signatures and surrogate data for p-value computation, and c) pattern set reduction which is conditional significance assessment to reject chance overlaps with background activity (rejecting false positive patterns in step b). to ensure that only meaningful and non-random synchronous spike patterns are retained as genuine neural ensembles (Torre et al., 2013; Quaglio et al., 2017). This method has been shown to be able to successfully detect synchronous neural ensembles during an instructed-delay reach-to-grasp delay task training in the macaque motor cortex (Torre et al., 2016). While original SPADE is primarily applied to detect synchronous spike patterns, it does not offer a significance evaluation of sequential patterns of neural activities. 3d-SPADE method is an extension of SPADE that addresses this limitation by providing a more comprehensive analysis of spatio-temporal patterns, allowing for a better understanding of the underlying neural dynamics (Stella et al., 2019).

5. SeqNMF[2]: This method has been developed to extract repeated large temporal sequential neural activity patterns in high-dimensional neural data. The seqNMF algorithm is based on matrix factorization techniques and is designed to extract informative and non-redundant sequences from neural data. Despite many neural ensemble detection algorithms which are based on pairwise spikes correlation, it uses a convolutional non-negative matrix factorization (convNMF) approach to

[1] Frequent itemset mining is a technique in data mining used to identify recurring patterns or sets of items within a dataset. It focuses on discovering combinations of items that frequently appear together in transactions or datasets.
[2] Sequential Non-Matrix Factorization

capture both synchronous and sequential events in the dataset. By decomposing the data into a set of factors, seqNMF can identify multiple neural sequences without relying on behavioral landmarks. One of the key features of seqNMF is its ability to identify long neural activity sequences in the presence of high level of noise or jitter in data. It has been successfully applied to various neuroscience studies, including the identification of neural sequences in hippocampal data and the analysis of abnormal sequence development in avian motor cortex (Mackevicius et al., 2019). Although, the algorithm provides a valuable tool for understanding the dynamics of neural activity and uncovering hidden patterns in complex datasets, it would be a time-consuming method when dealing with long-recording data. Moreover, seqNMF requires the hyper-parameters tuning step to optimize the algorithm performance. A generalized form of using convNMF with better parameters quantification has been proposed in PP-seq method by developing a Bayesian point-process model (Williams et al., 2020).

6. CAD[1]: This method focuses on capturing higher-order correlations within large-scale spike trains. It has the capability to extract both synchronous patterns and firing rate co-modulations, allowing for the detection of spike structures with flexible and diverse temporal configurations. CAD utilizes a statistical parametric test performed on neuron pairs, followed by an agglomerative recursive algorithm, to identify and statistically evaluate precise repetitions of spikes in the dataset. This method has been applied to different behavioral tasks and multiple brain areas including the hippocampus CA1, entorhinal cortex, and anterior

[1] Cell Assembly Detection

cingulate cortex and has shown that neural ensembles have different temporal structures in different brain areas (Russo and Durstewitz, 2017). In this study, we have employed CAD as the primary method for ensemble detection. This choice is motivated by CAD's unique capability to identify cell ensembles with varying correlation orders, encompassing both synchronized and sequential patterns. This versatility sets it apart from other methods, which are generally designed to detect only one specific category of ensembles. Furthermore, CAD utilizes a fast-parametric approach for ensemble detection, making it well-suited for the analysis of long-term recordings of neural data in terms of processing time. Additionally, CAD has been previously demonstrated to yield more substantial results in terms of identifying cell ensembles candidates compared to methods 3 and 5, when applied to the same dataset (Watanabe et al., 2019). This enhanced ability to detect ensembles can significantly contribute to a deeper comprehension of neural activities (detailed explanation of how CAD works can be found in the Method section).

It should be noted that development of such methods is one of current hot topics in computational neuroscience and application of such methods have been represented as an effective tool for analyzing neural activity associated with behavior and cognition (Tingley and Peyrache, 2020).

Although sleep was suggested as an integrated part of memory and learning process in the brain, different sleep stages have been demonstrated to play different roles in memory reformation and modification (Maquet, 2001). In the upcoming section, we will offer a concise overview of the different sleep stages, significant

local field potential events during sleep, and noteworthy studies and theories concerning how sleep contributes to the consolidation of various memories.

1.8. Sleep stages

Sleep is marked by a recurring pattern of two main types: Rapid Eye Movement (REM) sleep and non-REM sleep, which encompasses Slow Wave Sleep (SWS) and lighter sleep stages 1 and 2. In humans, the initial portion of the night, known as early sleep, is distinguished by a significant amount of slow wave sleep (SWS), while REM sleep dominates the latter half, referred to as late sleep (Aserinsky and Kleitman, 1953; Diekelmann and Born, 2010). Both SWS and REM sleep exhibit distinct patterns of electrical field potential oscillations and activity of neuromodulators. Slow oscillations (SO), spindles, and sharp wave-ripples (SWR) are the most prominent field potential oscillations during SWS, whereas REM sleep is characterized by ponto-geniculo-occipital (PGO) waves and theta activity. In the following, a brief description of some of these important events during sleep has been presented:

- Slow-Oscillations (SOs): These transient electrical waves are characterized in the frequency range of 0.5 to 2 Hz and mainly observed during deep sleep. They are typically generated by the interactions between thalamocortical circuits and the intrinsic properties of cortical neurons (e.g., membrane potential, membrane excitability, firing patterns, and synaptic integration mechanisms). The SOs consist of alternating phases of depolarization (up states) and hyperpolarization (down states) in the neuronal membrane potential (Steriade et al., 1993; Steriade, 2006).

During up state, neurons become active, fire action potentials, and exhibit increased cortical activity, along with a brief period of enhanced excitatory synaptic activity. In contrast, during down state, neuronal activity decreases, and the membrane potential becomes more hyperpolarized, characterized by reduced synaptic activity and relative neuronal silence (Battaglia et al., 2004; Massimini et al., 2004). SOs have been proposed to have association with the maintenance and regulation of sleep states, and are believed to play a role in the processes related to the memory consolidation and synaptic plasticity during sleep (Marshall et al., 2006; Johnson et al., 2010; Mölle and Born, 2011; Niethard et al., 2018).

- Spindles: Sleep spindles are periodic bursts of activity with waxing and waning oscillations at about 7–20 Hz that generally last 1 to 3 seconds. These oscillations are thought to be generated by the thalamic reticular nucleus which are transmitted to the cortex (Steriade et al., 1993; Kandel and Buzsáki, 1997). Spindles can be further categorized into two types: fast or low-voltage (10-20 Hz) and slow or high-voltage (7-12 Hz) (Skelin et al., 2019). Low-voltage spindles are thought to play a critical role in memory consolidation (Kandel and Buzsáki, 1997; Gais et al., 2002; Nishida and Walker, 2007; Johnson et al., 2010; Barakat et al., 2011). The process of fast-spindles nesting within SOs has been suggested to be important for memory consolidation during sleep (Johnson et al., 2010; Latchoumane et al., 2017; Niethard et al., 2018; Skelin et al., 2019). Additionally, calcium imaging study of cortical neurons revealed

significantly higher activation of putative pyramidal cells during fast-spindles coupled with SOs compared to the periods of solitary spindles suggesting the potential period for strengthening synaptic plasticity (Niethard et al., 2018). The strength of memory reactivation, as measured by Explained Variance and Template Matching, was found to increase with a higher density of fast-spindles. This indicates a direct correlation between memory reactivation strength and the density of fast-spindles (Johnson et al., 2010). The nesting of spindles within SOs were found to predict enhanced memory formation during the transition from childhood to adolescence. In adult humans, precise SO-spindle coupling were associated with memory consolidation promotion (Hahn et al., 2020). Spindles were suggested to facilitate the process of information transformation from the hippocampus to the neocortex in human brain, which is important for long-term memory storage (Andrade et al., 2011).

- Sharp-Wave Ripples: These are brief (30-120 msec) high-frequency oscillations that primarily induced by strong depolarizing inputs stemming from bursts of activity in the CA3 region of hippocampus which excite the CA1 neurons through the Schaffer collaterals, which are neural connections projecting from the CA3 to the CA1 cells (Buzsáki et al., 1992; Ylinen et al., 1995; Csicsvari et al., 2000). These waves are mainly generated during restful waking and non-REM sleep (O'keefe and Nadel, 1979; Karlsson and Frank, 2009; Buzsáki, 2015). Specifically, they are characterized by a fast ripple oscillation (100-300 Hz) superimposed by a

large amplitude slow-wave (0.1-1 Hz) and are suggested to be important in synaptic plasticity modification and memory stabilization processing (Carr et al., 2011; Rasch and Born, 2013; Roux et al., 2017). SWRs are strongly associated with memory processing. These waves are often associated with the reactivation and replay of neural patterns related to prior experiences or learning episodes. During slow-wave sleep, particularly in non-REM sleep, the hippocampus goes through a process of replaying these stored memories (Kudrimoti et al., 1999). This replay has been suggested to be possibly associated with the consolidation processing of episodic memories (Nádasdy et al., 1999; Dragoi and Buzsáki, 2006; Diba and Buzsáki, 2007). SWRs have been suggested to play a critical role in maintaining the stabilization of the spatial map (Roux et al., 2017). Research has demonstrated that prolonged SWRs in the hippocampus can enhance memory function (Fernández-Ruiz et al., 2019). Evidence has indicated different modulation patterns of neural populations during waking and sleep SWR, suggesting distinct roles associated with information processing for ripples during wakefulness and sleep (Jadhav et al., 2016; Tang et al., 2017; Abadchi et al., 2023).

- PGO-waves: These are characterized by strong bursts of synchronized activity during REM that spread from the pontine brainstem, primarily to the lateral geniculate nucleus and visual cortex (Lim et al., 2007). Increasing in density of PGO-waves after experience of a conditioned avoidance learning task has been suggested as a potential role of REM in

memory consolidation in humans (Datta, 2000). In addition, it has shown the linear correlation between successful fear extinction memory consolidating and the density of PGO waves during the sleep period following the traumatic stress (Datta and O'Malley, 2013).

- Theta Oscillations: These waves are generated in the range of 4-10 Hz and are characteristic features of tonic REM sleep primarily observed in rats and are predominantly observed in the hippocampus (Diekelmann and Born, 2010; Jacobs, 2014). A study on hippocampal neural ensembles represented the potential for sequential replay during REM sleep prior the experience. Additionally, they discovered a correlation between behavioral patterns and the subcortically driven theta rhythm, implying that reactivation during REM sleep might play a significant role in memory reprocessing during sleep (Louie and Wilson, 2001). Neural ensembles activations during peak or trough of theta oscillations in post-sleep were suggested to play a competing role in enhancing novel information and forgetting consolidated information associated with the episodic memories (Poe et al., 2000; Hutchison and Rathore, 2015). Moreover, variations in the coherency of theta oscillations between thalamo-cortical and limbic-hippocampal networks during REM have been proposed as a framework for synaptic consolidation (Robertson, 2009; Diekelmann and Born, 2010).

1.9. Motor memory reactivation during sleep

Numerous studies have highlighted the importance of sleep in memory

processing, including both declarative and non-declarative memories. Declarative memories, such as episodic memory, which primarily rely on the hippocampus in the initial phase of learning, undergo a gradual transformation from labile hippocampal information into more stable neocortical memory during sleep. The ultimate outcome is a semantic memory, which is well-consolidated and no longer dependent on the hippocampus (Marr, 1970; Wilson and McNaughton, 1994; McClelland et al., 1995; Buzsáki, 1996; Sirota et al., 2003). A previous study on hippocampal ensemble activity during a spatial navigation task reported a higher level of pairwise correlation among task-involved neurons during post-training sleep compared to pre-sleep and suggested the reactivation of memory during post-training sleep (Wilson and McNaughton, 1994). Researchers examining episodic memory observed sequential replay in both the hippocampus and the visual cortex during post-rest periods and they proposed that this simultaneous cortico-hippocampal replay might play a crucial role in consolidating declarative memories (Ji and Wilson, 2007). It has also been suggested that the sequential activation of spindles and SWRs is an instrumental phenomenon for the hippocampal-dependent memory (Peyrache et al., 2009).

Sleep is also believed to be essential in the consolidation of non-declarative memories like motor memory, in both human and animal studies (Fischer et al., 2005; Korman et al., 2007; Stickgold and Walker, 2007; Cheng et al., 2021). Having a certain amount of sleep was reported to improve motor skill performance (Walker et al., 2002). A study on rats' neuroprosthetic learning task found principle component reactivation of neural ensembles after motor skill training sessions with robust learning performance, specifically during slow-wave sleep (Gulati et al., 2014).

Increased slow-wave activities and coherency in neural ensembles during post-sleep were observed in the motor training experience, emphasizing the importance of SWS in novel skill consolidation (Gulati et al., 2017). Reactivation of rats' M1 neural ensembles during post-SWS, derived from PCA has been reported in skilled-reaching tasks (Ramanathan et al., 2015; Eckert et al., 2020). In addition, the quality of reactivation was correlated with the presence of spindles observation (Ramanathan et al., 2015; Eckert et al., 2020). Furthermore, the density of SO-coupled spindles has been reported to increase after motor learning experience, suggesting their potential role in offline skill processing (Kam et al., 2019). Also, the amount of nesting between spindles and SOs was proposed as the important mechanism of neural synchronization as part of the skill consolidation procedure (Silversmith et al., 2020). An optogenetic study of M1 neural activities revealed that nesting of fast-spindles coupled with SOs and coupled delta waves can play competing duties in the consolidation or weakening of novel skill-related neural activities (Kim et al., 2019). Spindles have also been suggested to be influential in facilitating cortico-striatal plasticity by interacting with SOs, strengthening the brain's capacity for skill consolidation (Lemke et al., 2021). While spindles have been linked to memory consolidation during SWS, higher changes in spindle power activity before strong reactivations during REM sleep suggested their significance in REM-related memory processing associated with skill learning (Eckert et al., 2020).

Despite many studies indicating the importance of SWS-reactivation in motor skill learning, REM sleep also has been proposed to be involved in the skill enhancement. REM sleep after experience of rotarod motor learning was also found

to influence critical changes in dendritic spines associated with network stabilization within mouse motor cortex (Li et al., 2017). The coupling reactivation of M1 neural ensembles during pre-rest REM and post-rest SWS has been suggested as a potential mechanism for faster skill acquisition in rats (Eckert et al., 2020).

Human studies have provided valuable insights into the role of sleep in the consolidation of motor memory and skill learning. During post-training sleep, neuronal activity in the motor cortex closely resembles the patterns observed during the initial phase of online skill learning, suggesting the potential contribution of sleep to the consolidation of motor skills (Rubin et al., 2022). Neural representation of online skill learning showed within the compressed activation pattern during sleep (Eichenlaub et al., 2020). It was unveiled a significant positive correlation between improvements in motor performance and the amplitude of spindles as the predictor of skill consolidation strength (Barakat et al., 2011). Moreover, it has been suggested that offline performance gains associated with memory reactivation during sleep in humans are supported by altered functional activity in key cognitive and motor networks (Cousins et al., 2016). Additionally, the consolidation process appears to be differentially mediated by both REM sleep and SWS, highlighting the complex and multifaceted nature of sleep's role in the enhancement of motor skills (Cousins et al., 2016).

1.10. Hippocampus involvement in motor skill learning

Traditional studies suggested that the Hippocampus is not required in learning of procedural memories like motor skill learning (Squire and Zola, 1996; Gould et

al., 2002). However, recent studies of human brain have proposed the potential involvement of the Hippocampus in motor sequence learning tasks. An fMRI study of neural activity in the Hippocampus and the Striatum during an implicit oculomotor sequence learning task revealed a linear correlation between the amount of activity in both regions and the between sessions gain performance. This suggests the significance of both regions in procedural memory processing and possibly consolidation (Albouy et al., 2008). In addition, this research uncovered that the hippocampal involvement in learning, observed during training in fast learner groups, predicts the improvement in behavioral performance observed 24 hours later, suggesting the hippocampal activities during initial training phase may influence following offline memory processing (Albouy et al., 2008). While this study indicated the Hippocampus' contribution in the early stages of the training phase, another human fMRI study on implicit motor sequence learning demonstrated the involvement of this region in both skill exploration and skill consolidation phases. Additionally, they found a correlation between Hippocampal activation and learning performance (Gheysen et al., 2010).

1.11. Theory and Hypothesis

Overall, Motor memory consolidation during sleep is a crucial process for skill promotion and the neural circuit plasticity associated with procedural learning. Previous reports have underscored the role of sleep in the memory reactivation associated with skill enhancement. These studies have primarily relied on PC-reactivation analysis, particularly focusing on the exploration of the first principal

component (PC1), which characterizes a unique neural ensemble dynamic during motor actions (Gulati et al., 2014; Ramanathan et al., 2015; Eckert et al., 2020). While it has been suggested the involvement of various neural dynamics during motor execution (Rokni et al., 2007; Liberti III et al., 2016; Peters et al., 2017), it remains unclear whether other types of neural activities associated with motor learning significantly contribute to the memory processing during offline periods. Moreover, the majority of analyses regarding the reactivation of motor memory have been based on PC-reactivation analysis, which indeed demonstrates the association between the principal components of neural ensembles during sleep and behavior. However, it does not directly represent the spatiotemporal replay/ reactivation of the ensemble during sleep. Therefore, the interpretation of reactivation is complex and requires further validation and evaluation. Furthermore, it is poorly understood whether the integration of episodic knowledge and motor information during sleep correlates with skill performance enhancement. Therefore, the problems addressed three main problems:

- first, to develop a more comprehensive understanding of the neural dynamics associated with the motor learning during online phase of training.
- second, to explore more details of sleep-associated with motor memory reactivation helping with the skill enhancement. This entails an investigation into the relative contribution of coupling level between SOs and spindles to reactivation of the motor cortex.
- third, to examine the potential interactions between the primary motor

cortex and the hippocampus which may correspond to skill learning performance

Hypothesis: The main hypothesis behind this research study is that an unsupervised machine learning reactivation/replay framework can detect reliable neural activity patterns that contribute to the online and offline learning periods. The proposed study seeks to answer the following three key questions:

1. Are spatiotemporal M1 cell ensembles involved in motor learning replayed during sleep?
2. What is the relative contribution of fast-spindles coupled with SOs to the reactivation M1 cell ensembles with different online dynamics?
3. Is there a tendency for M1 neural ensembles linked to an online sensorimotor skill learning task to be reactivated/replayed during hippocampal SWRs in SWS, and if so, is this tendency correlated with learning outcomes?

By addressing these questions and testing the main hypothesis, this research aims to provide a comprehensive understanding of the correlation between neural information processing during online and offline periods.

2. Methods

This part describes the recording information, behavioral procedures, and data analysis steps. The dataset was recorded by (Eckert et al., 2020) and a summary of procedure and recording procedures (section 2.1-2.5) has been explained here, however data analysis part has been represented in detail.

2.1. Animals and experimental design

In this study, we analyzed data from previously recorded dataset of 4 adult male Fisher-Brown Norway rats trained on a motor skill task (Eckert et al., 2020). To provide a concise overview, each daily recording session was composed of an initial 3 hr resting period before the task (rest 1), followed by 30 min of skill training on the single pellet reaching task and concluded with a 3 hr resting period after the task (rest 2) (see Figure 1.A). A hyperdrive, which included an array of 12 tetrodes and 2 reference electrodes, was implanted on deep layers of the forelimb area of primary motor cortex (coordinates: 1.00 mm anterior, 2.5 mm lateral to bregma) and neurons' spike activity was recorded throughout the entire 6.5 hr recording session. In addition, hippocampal local field potential (LFP) was recorded by a twisted bipolar electrode implemented in the dorsal hippocampus. EMG activity was measured a wire implanted into the neck muscle (Eckert et al., 2020).

2.2. Data acquisition

The recording process utilized the digital Cheetah SX data acquisition software by Neuralynx (Boseman, Montana). Spike recordings were obtained with bandpass

filtering (600–6000 Hz) and sampling at 32 kHz. A reference electrode was positioned in the cortical white matter for tetrode recordings. Simultaneously, LFP and EMG signals were filtered (0.1–1000 Hz), sampled at 2 kHz, and referenced to a cerebellar skull screw (Eckert et al., 2020).

2.3. Behavioral task

During the task phase, rats were situated within a custom-designed polycarbonate enclosure tailored specifically for the single-pellet reaching task (Whishaw and Pellis, 1990). This container featured a front opening with a 1.5 cm slot, enabling the rats to reach through and retrieve a 45 mg food pellet located 1.5 cm away on a 3 cm high shelf. Task performance was recorded using a high-speed infrared camera capturing at 200 frames/sec. An infrared beam at the front of the cage detected reaching actions. The animals engaged in approximately 60 trials during the 30 min session. During the intervals between trials, a sliding door was used to obstruct access to the slot with aim of regulating both the duration between trials and the number of trials carried out each session. In rest periods, rats relaxed inside a flowerpot furnished with towels, while their activity was monitored and recorded using an infrared security camera. The rats also underwent a one-week habituation period to the recording process before commencing training on the reaching task. It should be noted that rats had no prior experience or training in the specific skill being tested, except for the initial assessment to determine their paw preference. All animals had at least 7 days before training started where they went through the entire recording procedure (including pre-rest, task, and post-rest), except for the fact that

they didn't do skilled reaching during the task part (Eckert et al., 2020).

The dataset included the following information:

- Task session: animal paw advance, pellet grasping and returning timestamps.
- Rest (1 and 2): sleep stages (REM, SWS, motionless), events timestamps including slow-oscillations (SO), SO-uncoupled spindles (isolated spindles), SO-coupled spindles, and sharp-wave ripples (SWR).
- Whole recording: neurons spike times (Table 2 shows the number of recorded neurons at each training day).

Table 2. Number of recorded neurons per training day (from Eckert et.al 2020)

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
rat 1	27	51	57	37	50	81	47	45	43	59	65	63	67	67	39					
rat 2	19	19	46	34	28	21	33	33	31	47										
rat 3	50	58	58	84	76	72	61	63	70	49	70	62	74	74	70	71	75	52	62	65
rat 4	21	22	41	36	37	21	42	25	14	14	24	19	16	19	26					

2.4. Behavioral assessment

Reaching behaviour was scored manually by the experimenter using the high-speed video recording. The evaluation of animal performance was conducted based on two metrics called as success rate and duration of reach trials. The success rate measured the ratio of successful trials to the total number of attempted trials. A successful trial was defined as one in which the rat successfully grabbed the pellet, brought it back, and consumed it. Any other trials, such as misses or instances where the rat grasped the pellet but dropped it during retrieval, were categorized as unsuccessful. In addition, the duration of reach performance was achieved by

measuring the average time difference between the start of reaching movement (paw advancing) and the end of the reaching movement (paw returning time) across trials in each session. It's important to note that only single-attempt trials were considered in this measurement.

2.5. Sleep stages identification

REM sleep was classified within rest epochs using theta power, delta power, and EMG power measurements. Theta and delta power were computed from the hippocampal LFP while EMG power was extracted from the EMG signal. A threshold (greater than 3 standard deviations) was applied to determine REM sleep epochs. SWS periods were based on upper delta power (Eckert et al., 2020).

2.6. Spindles and SOs identification

In order to detect low-voltage spindles, the LFP signal was filtered between 10 and 20 Hz. Peaks in the power signal exceeding 1.5 standard deviations, were identified and expanded down to 0.75 standard deviations to establish the start and end timestamps. Successive events within an interval of 100 msec were merged into a single event. SO events were obtained by filtering the LFP signal within the delta band (1–5 Hz) and by comparing it with a 35 msec time-shifted signal. Peaks that exceeded a threshold criteria of 3 standard deviation (sd) above the average signal were recognized as the SO events. The coupling of spindles with SOs was performed by connecting each spindle to its nearest SO and examining if it was observed in a window of 500 msec (Eckert et al., 2020).

2.7. SWR detection

The Hippocampus LFP signal was bandpass-filtered within the frequency range of 100 to 250 Hz. After rectification, the mean and standard deviation (s.d.) of power were calculated. Events displaying power levels significantly exceeding the mean + 2.5 sd were identified as SWR events (Eckert et al., 2020). In addition, events lasting more than 150 msec were omitted, and events separated by intervals of less than 25 msec were combined into a single event.

2.8. Cell ensemble detection

Cell ensembles were detected by the unsupervised CAD algorithm proposed by (Russo and Durstewitz, 2017) during combined task and sleep spiking data. The CAD algorithm presents a comprehensive approach for extracting spatiotemporal neural patterns with arbitrary lag structures, enabling unbiased analysis without the need for templates. In a brief overview of CAD algorithm, it combines statistical assessment of spike count distributions with an agglomerative clustering approach to identify higher-order cell assemblies exhibiting significant patterns of neural activity. In detail, this algorithm consists of two main steps as follows:

1. Statistical assessment of spike count distributions:

   The CAD algorithm begins by evaluating the joint spike count distribution of neuron pairs A and B to assess their independence. Binary spike count series are constructed by discretizing the spike time series using a user-defined bin width (Δ). Assuming independence, the joint spike count (#AB,l) at time lag 'l' follows a hypergeometric distribution. To account for non-stationarities

resulting from local firing rate changes, the series is divided into binary subseries, and a difference statistic $\#_{ABBA,l} = \#_{AB,l} - \#_{AB,-l}$ is calculated to eliminate local non-stationarities. The statistic Ql, derived from $\#_{AB,l^2} \Big/ \hat{\sigma}^2{}_{ABBA,l}$ approximates the F-distribution and serves as a parametric test statistic for independence, mitigating the influence of local non-stationarities.

2. Agglomerative clustering step:

   After assessing the independence of neuron pairs, an agglomerative clustering algorithm is employed to merge significant pairs into higher-order cell assemblies. At each agglomeration step, previously merged unit sets are treated as single units with defined activation times. Pairwise tests, following the same procedure as for the single units, are conducted on these unit sets, allowing for the detection of higher-order dependencies. Each pair is tested at different time lags, and if found significant at multiple lags, only the one with the minimum p-value is retained. The agglomeration process continues until no more significant relationships among sets and single units are detected. Importantly, CAD is capable of identifying cell assemblies with varying population sizes. Subsets within larger sets are discarded, and the procedure is repeated for different bin widths. The temporal precision of each cell assembly is determined by the bin width associated with the lowest p-value. All statistical tests are performed at a user-specified Bonferroni-corrected significance level.

CAD can be configured to detect cell ensembles at different temporal resolutions. It is worth noting that ensembles detected at different bin sizes may

exhibit some degree of overlap. In such cases, a pruning approach can be employed to retain the most reliable or larger-sized ensembles across different temporal resolutions. Current pruning suggestions can be summarized as follows:

1. Removal of smaller ensembles within larger ensembles: One approach to pruning involves eliminating smaller cell ensembles that are subsets of larger ensembles. This ensures that the analysis focuses on the most prominent neural assemblies, which may be more relevant for understanding neural coding and information processing.
2. Distance Clustering Approach: An alternative pruning strategy employs a distance-based clustering approach. This method aims to find the most frequent or statistically significant ensembles based on their proximity or similarity among other neural activity patterns. By doing so, it emphasizes the preservation of neural assemblies that are most likely to convey meaningful information or exhibit consistent behavior across different conditions or time points.

CAD Inputs and outputs: The algorithm requires spike data, temporal resolution, and maximum lag constellation as the primarily inputs. The main output of CAD provides a list of cell assemblies including the following information:

- neuron members: these are neuron IDs involved in the detected ensemble. The ensemble's population size is defined as the number of neuron members.
- lag structure: this represents the firing delay of ensemble members with

respect to the first neuron's firing time (lag[z] represents the activation delay between neuron[1] and neuron[z+1], z=1,…, ensemble population size).

- bin size: this is the temporal resolution of detected assembly.
- activation time: Times when the cell assembly is significantly detected across the recording time (expressed in the unit of binned spike trains).
- activation power: A measure of the assembly's activation across recording time. It can be expressed in various ways, such as binary, partial, hybrid and with considering ensemble's lag structure or first member firing (point processing) at each activation time. The binary approach examines whether if cell assembly is activated or inactivated. The partial approach measured ensemble's activity power at each time point by averaging the number of shared spikes across member neurons. The hybrid approach is a regularized extension of the partial approach, mediating the effect of inactivated member neurons at the time of ensemble activation.
- Pvalue: A vector containing pvalues of detected ensembles at each agglomerative step. In the other word, pvalue[z] is the pvalue of the statistical test when adding neuron[z+1] to the neuron[1:z].
- Signature: Array of two entries (z,c) where the first entry represents the number of neurons participating in the ensemble (population size), and the second entry represents the number of ensemble occurrences.

2.9. Cell ensemble activity measurement

To quantify cell ensemble activity through the time, we utilized the binary activation time series for each cell ensemble, based on the designated detection bin size. In this time series, a value of 1 denoted ensemble detection, while a value of 0 indicated the absence of detection. This binary activation approach was chosen to mitigate the influence of local non-stationarities that might lead to variations in the spike counts among ensemble members during recording times. Furthermore, we defined the activation time as the moment of the first neuron firing bin edge, serving as the ensemble activation driver unit in any arbitrary internal configuration. In essence, our approach imposed stringent criteria on ensemble activity to minimize the impact of abrupt changes in neural firing rates and differences in detected ensembles internal firing structures.

2.10. Capturing cell ensembles dynamics surrounding reach behavior

To capture the dynamics of cell ensembles during the reaching behavior, we opted for a time window of ±5 sec centered around the pellet grasping time within each single-attempt trial. We constructed peri-event time histogram (PETH) for each cell ensemble using the binary activity time series. The choice of PETH length was based on the analysis of activation dynamics in a window of approximately 10 times larger than the average reaching trials duration (1±0.019 sec, mean ± sd across animals) to ensure a comprehensive examination of neural activity changes associated with the reaching movement.

We then applied a two-step preprocessing procedure to the PETHs. First, we

used LOESS regression to smooth the data and reduce the noise impacts. Next, a filtration step was conducted to eliminate PETHs that exhibited minimal correlation with the reach behavior. This process involved the establishment of a distribution comprising the maximum probability of activation values derived from all PETHs. We next applied a threshold criterion to this distribution, specifically set at the 75th percentile to select PETHs to select ensembles placed in upper 3rd quantile range as the potential reach-related candidates. The thresholding value stemmed from the fact that the overall duration of reaching trials was so much smaller than the entire recording session (approximately 2%). As we ran CAD on the whole session, we expected to find more cell ensembles activated outside of the task execution period. As a result, numerous detected ensembles exhibited low modulation with the reaching period and were thus excluded from further analysis.

To identify potential similarities among cell ensembles' dynamics around reaching behavior, Kmeans clustering with Euclidean distance similarity metric was applied to the preprocessed and standardized PETHs. The optimal number of clusters was determined using the Silhouette metric by varying K in the range of (2, 20) and finding the value of K corresponding to the maximum Silhouette value. To mitigate potential bias from specific dynamic types or individual rats, we conducted initial clustering on a per-rat and per-day basis. we selected the most frequently occurring value of K across sessions to establish a fixed number of clusters across training days for subsequent analyses.

2.11. Sleep reactivation analysis

Our reactivation analysis aimed to assess the activation strength of reach-related cell ensembles by comparing rest 1 and rest 2 sleep epochs. We quantified activation strength during sleep stages by examining the number of activations in rest 1 and rest 2. However, it's important to note that rest 1 and rest 2 differed in the duration of SWS and REM sleep. To ensure comparability and control for the expected increase in counts due to longer durations, we normalized the number of activation times by the respective stage's duration to accurately account for variations in detection counts based on the length of the sleep stage under consideration. We performed reactivation analysis for each of reach-related cell ensembles independently by comparing median activation rate of ensemble members activation rate derived from each recording session.

2.12. Cell ensemble activity around spindles

We first compared the activation strength between spindle periods and non-spindle periods during SWS. Here, both isolated spindles and SO-coupled spindles were combined and treated as a singular entity. The average activation count within ±1 sec of center of spindle events was measured. Next, we selected random 2-second events between spindles during SWS, ensuring that these events did not overlap with spindles. We then measured the average activation count of ensemble activation during these non-spindle events. This process was repeated 1000 times to create a distribution of activity during non-spindle events. We compared the resulting shuffled distribution, which represented activation strength during inter-spindle

periods, with the average activation during spindles. Additionally, we investigated the activation strength between isolated spindles and SO-coupled spindles by comparing the ensemble's average activation count around ±1 second of the spindle events' center.

2.13. Cell ensemble modulation with SWRs

To explore potential modulation between M1 ensembles reactivation and hippocampal SWR events, we examined peri-ripple activity for each cell ensemble within a window of ±150 msec centered around the middle point of sleep-associated ripples. We used a temporal resolution of 10 msec for this analysis. Then, 1000 peri-random events were created by selecting random points between SWRs and creating the average activity profiles with duration of 300 msec. Importantly, these randomly generated events were deliberately designed to have no temporal overlap with actual ripple events. We derived total activation within the peri-ripple time histogram and peri-random time-histogram to determine ensemble modulation during ripple events. The observed modulations were classified into three categories: SWR-increased, SWR-suppressed, and SWR-no preference (n.p). Specifically, if the metric measured for the SWRs exceeded the 95th percentile of the distribution derived from the shuffled random events, it was labeled as SWR-increased. Conversely, if the metric fell below the 5th percentile of the shuffled distribution values, it was categorized as SWR-suppressed. Otherwise, it was determined as SWR-no preference (n.p).

To understand the prevailing suppression or activation modulation mode across recording sessions, we calculated a Modulation Index defined as the ratio of the

number of SWR-increased ensembles to the sum of both increased and suppressed ensembles.

$$Modulation\ Index = \frac{\#\ SWR_{increased}}{SWR_{increased} + SWR_{suppresed}}$$

2.14. Temporal coupling between SWR and SO-spindles

To examine changes in the timing relationship between SWRs and SO-spindles over the course of learning days, we connected each SWR with the nearest SO-spindle by calculating the absolute delay amount between the SWR center and the SO-spindle onset times for each recording session. We then generated a histogram of coupling values in the range of (-2,2) sec from the spindle onset time, utilizing the temporal resolution of 2 msec. The histogram was smoothed with a Gaussian filter having a kernel size of 50 msec. The delay between SWRs and SO-spindles, represented for each recording session, was determined by identifying the time point corresponding to the location of the maximum percentage within the smoothed histogram.

2.15. Statistical analysis

We employed non-parametric statistical tests for statistical analyses otherwise specified. The main reason for selecting non-parametric tests was that our results did not necessarily follow the assumptions of normal distribution and homogeneity of variance. Additionally, nonparametric tests are more robust to outliers, making them a suitable choice for our analysis. Pairwise comparison was conducted using the Wilcoxon signed-rank test while Kruskal-Wallis test was used to compare more than

two groups. Post-hoc multiple comparisons were carried out using Dunn's test. Bonferroni Correction was applied to adjust pvalues for multiple testing. The statistical significant threshold was set as $p< 0.05$ (In all figures, asterisks indicate the level of pvalue range: * $0.01<p<0.05$, ** $0.001<p< 0.01$, *** $p< 0.001$). All figures represent mean ± sem (if eligible) unless it was indicated otherwise.

2.16. Data analysis hardware/ software

We used the Python version of CAD available in the Elephant package- an open-source, community-centered library for the analysis of electrophysiological data in the Python programming language (Denker et al., 2018). Cell ensemble detection was performed on the Cedar cluster system provided by the Digital Research Alliance of Canada (former ComputeCanada). The rest of data analysis steps were executed on the Polaris cluster system provided by the University of Lethbridge and a workstation system (Intel 11th Gen Intel(R), Core i5-11600K @ 3.9 GHz, RAM 32 GHz). All programming and data analysis steps were carried out using Python 3.10.

3. Results

3.1. Behavioral results

We explored M1 neural activity of four rats in a dataset designed for skill learning and recorded for approximately 6.5 hr, including 3 hr pre-sleep, 30 min task, and 3 hr post sleep (Figure 1.A, see Methods and (Eckert et al., 2020) for more details). We evaluated behavioral performance using two metrics called success rate and reach duration. When considering changes of success rate across training days, it was indicated rat 1 and rat 2 exhibited notably very low success rates which then experienced a rapid increase beginning from day 4. Conversely, the other two rats performed a more gradual learning pattern characterized by a moderate learning rate during the initial phase of training. Consequently, we classified rat 1 and rat 2 as rapid learners and referred to the other two rats as gradual learners (Figure 1.B, see also (Eckert et al., 2020)). In addition, we explored how quickly animals performed the tasks across training days (Figure 1.C) and observed difference between rapid and gradual learners. Rapid learners demonstrated the ability to perform the reaching task more quickly on the later days of training (day 10-15 in all rats, except for rat 2: day 5-10) of training compared to earlier days (day 1-5 in all rats) whereas gradual learners did not show significant trend across training days (Figure 1.D, Wilcoxon signed-rank test, rapid learners (D(i)): $p=0.0039$, gradual learners (D(ii)): $n=10$, $p=0.1309$).

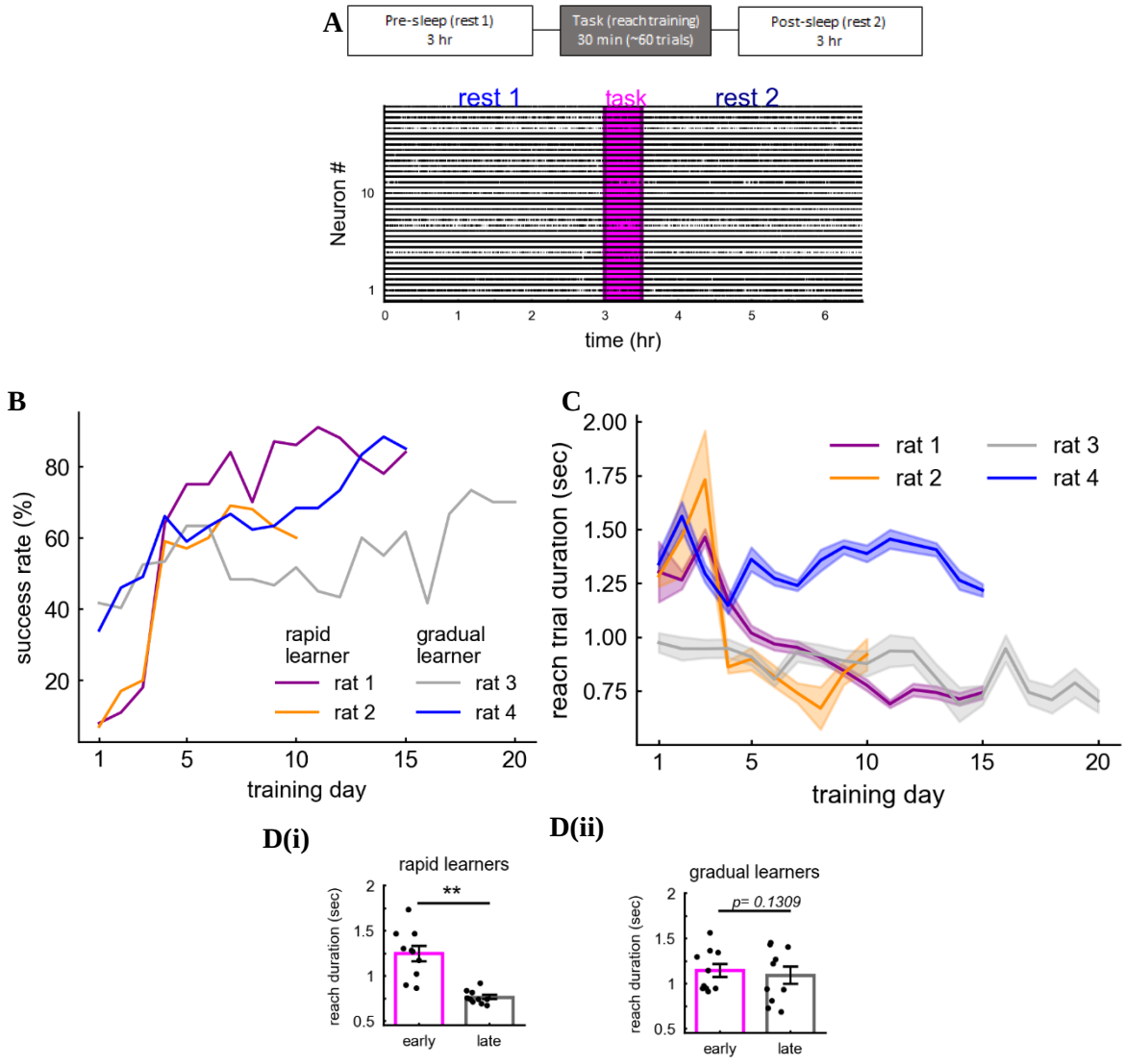


Figure 1. Experimental design and behavioral performance. A, experimental procedure during each recording session. The spiking activity of the primary motor cortex was recorded by tetrode recording during the entire session. B, success rate changes across learning days. Rat 1 and 2 performed low success rate followed by a significant increasing at day 4 and then gradually increasing. However, rat 3 and 4 showed a moderate success rate from the early phase of training followed by gradually changes during different days. We called first two rats as rapid learners and two other rats as gradual learners (adapted from (Eckert et al., 2020) with permission). C, changes of reaching trials duration (lag between paw advance (reach onset) and paw returning (reach offset)) across training days (mean ± sem across trials). D, comparison of reach trials average duration between training phases (early and late) for different animal groups. D(i): rapid learners (Wilcoxon signed-rank test, p=0.0039, mean ± sem). D(ii): gradual learners (Wilcoxon signed-rank test, p=0.1309, mean ± sem).

3.2. Cell ensembles detection at different temporal resolutions

To comprehensively detect cell ensembles activated throughout the entire recording process, we conducted unsupervised CAD method for each recording session (total number of sessions = 60, each session duration ≅ 6.5 hr). We systematically evaluated cell ensembles in 10 different bin sizes ranging the intervals of [3, 5, 10, 25, 50, 65, 75, 90, 100] msec (resulting in total of 60 session × 10 bin sizes = 600 datasets). The primary motivation for detecting cell ensembles at different bin sizes was to accommodate user-defined temporal resolutions and to identify potential sequential replay events during sleep with varying compression or extension rates.

Our initial observations showed a diverse array of ensembles characterized by varying firing structures. These included synchronous and asynchronous (or sequential) activation. Synchronous activation refers to the simultaneous firing of all ensemble neuron members within the same bin (Figure 2.A(i)). However asynchronous structures were observed within two different structure as: a fixed lag level between any two consecutive neurons (Figure 2.A(ii)) or an arbitrary relative delay among neuron members (Figure 2.A(iii)). Our quantification analysis of relation the between number of recorded neurons and the resulting number of detected cell ensembles demonstrated an exponential growth pattern, indicating that as the number of neurons increased, the number of detected cell ensembles grew exponentially (Figure 2.B, linear regression: $\log(y) = ax + b$, p=1.88 × 10^{-22}, r^2=0.8084).

Considering the proportion of ensembles at different bin sizes, our observations

revealed a positive correlation between the temporal bin size and the number of identified cell ensembles. Specifically, larger bin sizes yielded a higher count of detected ensembles, primarily due to the increased likelihood of neural pairing (Figure 2.C).

Additionally, we explored the composition of cell ensembles in terms of population size and activation lifetime at different temporal resolutions. Taking all ensembles as a single group, our results demonstrated that the majority of detected ensembles were indeed in larger bin sizes and with population size between the range of (2-6) neurons (Figure 2.D(i)). Additionally, it was indicated that smaller bins tend to yield ensembles with few members, while larger bins generate more extensive ensembles (Figure 2.D(ii)). Building upon previous research indicated that cell ensembles in distinct brain regions exhibit different firing patterns (Russo and Durstewitz, 2017), our investigation of M1 neural activities suggested that the majority of the identified cell ensembles demonstrated a short activation lifetime, characterized by either zero-lag correlation or low-order correlation (Figure 2.E(i)). Indeed, when analyzing cell ensembles at small temporal resolutions, sequential structures with short lifetime were dominant however in larger bins, most ensembles exhibited the synchronous activation pattern (Figure 2.E(ii)). We used the ensembles' activation times to determine their dynamics and modulation during both online and offline periods. An example of a detected cell ensemble during a recording session has been shown in (Figure 2.F).

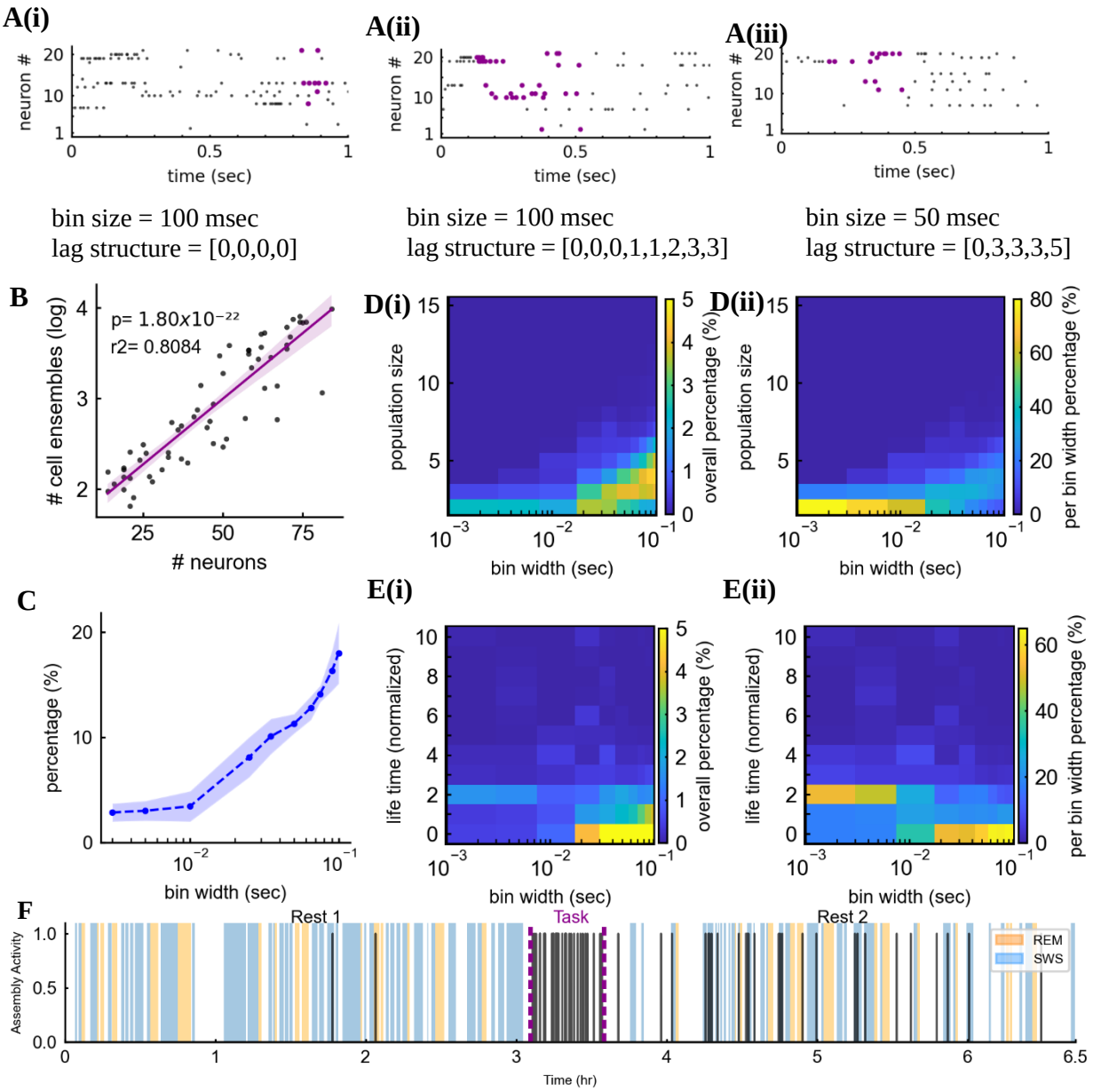


Figure 2. Cell ensemble detection. A, examples of detected cell ensembles' firing structure, A(i): synchronous (zero-lag correlation), A(ii): sequential activation with fixed lag structure, A(iii): sequential activation with arbitrary lag structure between member neurons. B, percentage of number of detected cell ensembles across different bin sizes (n=4 rats, mean ± sem). Larger bins produced more ensembles. C, number of detected cell ensembles across different recorded neurons (n=60 sessions, scatter plots). Linear regression fit on logarithm of cell ensembles numbers (y) and number of neurons (x): $log(y) = ax + b$, p = 1.80 x 10^{-22}, r^2=0.8084). D, histogram of cell ensembles population size (number of neuron members) across different bins, normalized across total number of ensembles (D(i)), and across detected ensembles per each bin (D(ii)). E, histogram of lifetime of cell ensembles (the lag between first and last neurons firing) activation, normalized by the total number of ensembles (E(i)), and by the detected ensembles per each bin (E(ii)). F, an example of a cell ensemble activity during a recording session (black vertical lines indicate the moment of activation)

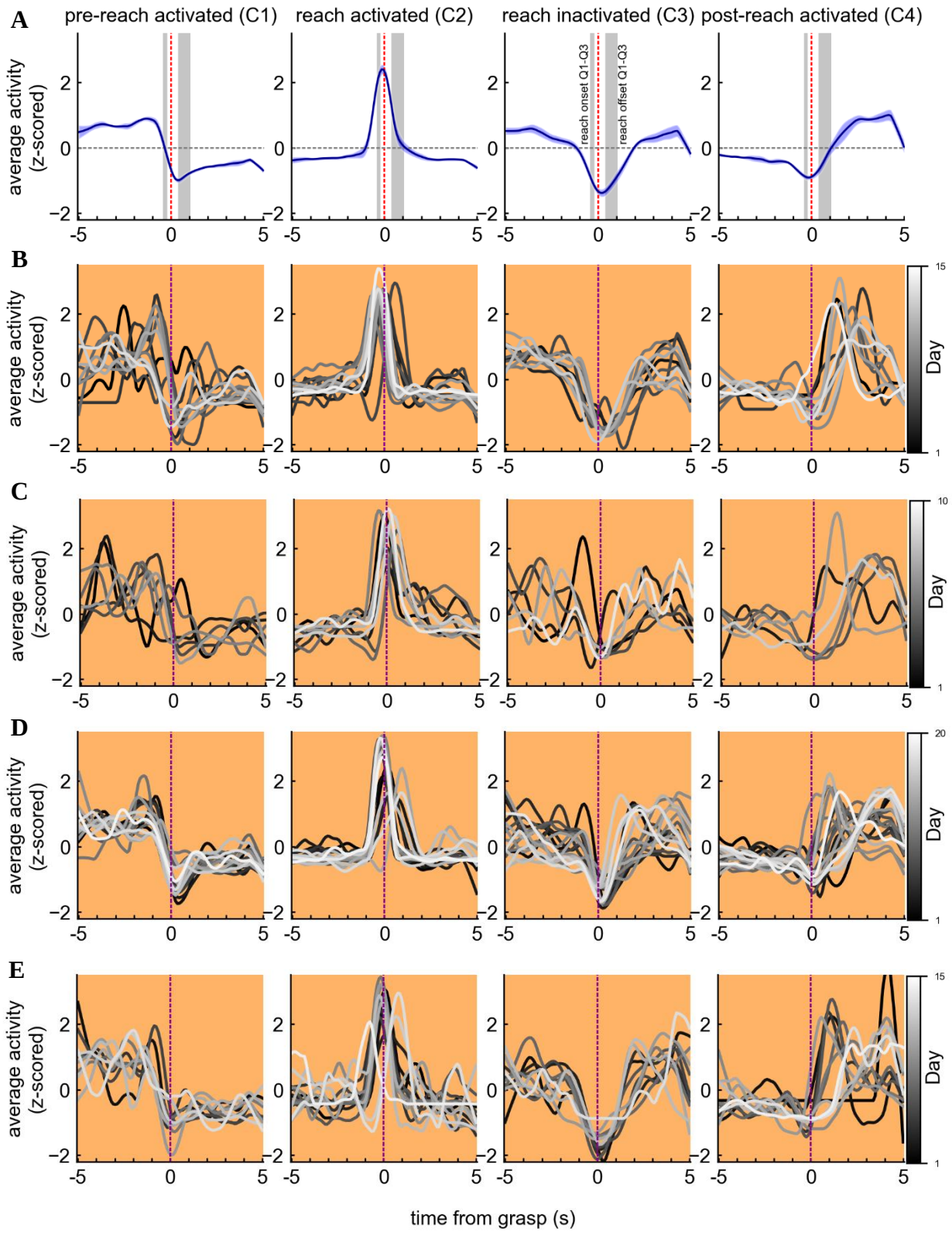
3.3. Different cell ensembles activation dynamics around reaching behavior

Motor cortex neural ensembles exhibit diverse and intricate dynamics during movement execution (Rokni et al., 2007; Peters et al., 2017). Previous analysis of neural activities during the reach-to-grasp task using PCA reported a specific pattern with the excitation modulation around the time of grasping (Eckert et al., 2020). Here our goal was to explore other significant dynamics associated within this task. Due to detecting multiple cell ensembles, we employed Kmeans clustering on their PETHs during the online training period to identify similar dynamics (see Methods). We conducted the clustering separately for cell ensembles detected at different temporal resolutions. Note that we observed highly fluctuating and low-quality PETHs when using ensembles detected in smaller bin sizes. Indeed, these ensembles were affected by high levels of noise, rendering clustering ineffective and providing limited meaningful results. Additionally, there was a significant degree of overlap among ensembles detected with larger bins, leading to nearly identical results. Consequently, we focused our analysis on ensembles detected at the largest bin size (10000 msec), which not only represented the majority of identified ensembles but also included the largest-size cell ensembles (see Figure 2.D(i)).

The clustering analysis of cell ensembles detected at the temporal resolution of 100 msec yielded four distinct dynamics during the reaching behavior and specifically relative to the grasping time (Figure 3.A). These clusters were designated as pre-reach activated (C1), reach-activated (C2), reach-inactivated (C3), and post-reach activated (C4). In the context of these clusters, the ensembles within C1 demonstrated preparatory-like properties with elevated activity levels preceding the

reaching onset, followed by a rapid decrease in activity once the reach was initiated and maintained at a lower level thereafter. C2 ensembles exhibited sharp increasing activity during the reaching phase, peaking around the moment of pellet touching and followed by rapid decline of activation. This pattern of activity resembled the PC1 dynamic identified by PCA in the previous study that utilized the same dataset (Eckert et al., 2020). C3 ensembles, in contrast, showed a suppressive modulation during the movement execution compared to outside of task period, and the minimum activation (largest suppression) was observed around pellet grasping time. Lastly, C4 ensembles indicated increasing activity after reach onset, with the initiation of this heightened activity coinciding with the grasping moment. Importantly, in the majority of training sessions involving all rats, we consistently identified these four distinct dynamics (Figure 3.B-E). We also explored how the number of clusters was influenced by learning over multiple days. This analysis aimed to enhance the robustness of our results by accounting for potential effects of the learning process across different days. Statistical analysis revealed no significant differences in optimal K values (resulted by Silhouette metric analysis) across different phases of training subdivided by day 1-5 (early), day 5-10 (middle) and day 10-15 (late) (for rat 2 we divided it into 2 phases as early and late) (Figure 3.F, Kruskal-Wallis test, $p=0.3939$). Note that we excluded the outlier K values using the 95^{th} percentile threshold for finding the most frequent K across sessions. We found that only a single day was removed (rat 2, day 10) although there was no significant difference of statistical results considering outliers or not. We then selected the mode of K values across recording sessions as the fixed number of clusters, which was K=4 (Figure

3.G, Silhouette score at K=4: 0.36 ± 0.01; mean ± sem across recording sessions). Additionally, we did not find significant differences in the quantity of detected cell ensembles among the reach-related groups (Figure 3.H, Kruskal-Wallis test, p=0.272). In summary, our analysis revealed four different neural activity profiles whose general time-domain properties were almost similar across the learning days.


A
pre-reach activated (C1)
reach activated (C2)
reach inactivated (C3)
post-reach activated (C4)
reach onset Q1-Q3
reach offset Q1-Q3
average activity (z-scored)
B
C
D
E
Day
time from grasp (s)

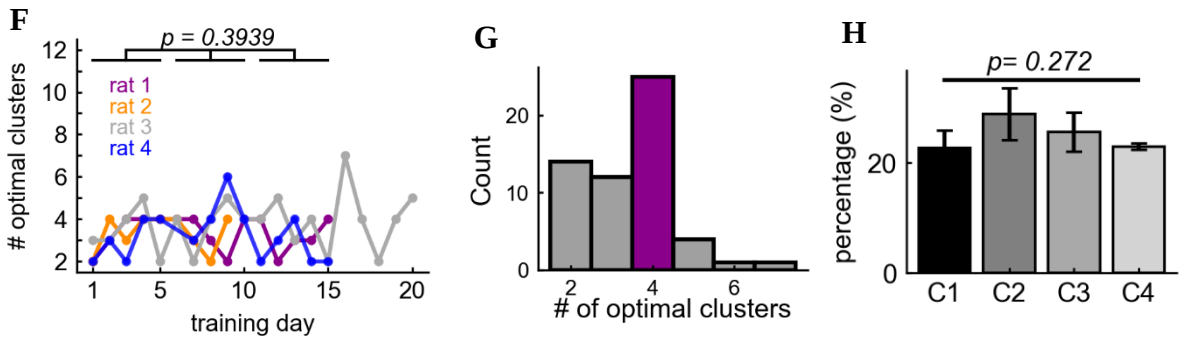


Figure 3. Dynamics of cell ensembles around reaching behavior. A, Four main dynamics resulted from Kmeans clustering labeled as pre-reach activated (C1), reach activated (C2), reach inactivated (C3) and post-reach activated (C4) from left to right (n=4 rats, solid blue line indicates average ensembles' PETH across rats, shaded region shows sem). left-sided gray bar shows Q1-Q3 range of the reach onset time relative to the grasping time, while right-sided bar represents same range for the reach offset time. B-E, clustering results per day for each rats 1-4, respectively. Each solid line represents the average PETH across detected ensembles for a single day. F, Variations of the optimal number of clusters (K) across different phases of training (days 1-4, days 5-10 and days 10-15) (Kruskal-Wallis test, p=0.3939). The results after the outlier removal-which only day 10 in rat 2 was removed- has been represented. G, histogram of number of optimal clusters resulted by Kmeans clustering performed on the individual recording session and evaluated by the Silhouette metric. Mode of the K values (magenta bar) was selected as the fixed optimal number of clusters to have the same number of groups across the days. H, percentage of number of cell ensembles at different clusters (mean ± sd across rats, Kruskal-Wallis test, p= 0.272).

3.4. Enhanced activation during post-sleep compared to pre-sleep

Reactivation of M1 neural ensembles during post-training sleep is thought to be important for skill consolidation (Gulati et.al, 2014, Ramanathan et.al 2015, Eckert et al. 2020). However, these findings were primarily based on PCA-associated with reactivation, specifically PC1, which indicated reach-activated cell ensembles (C2) in our study. Given the emergence of additional reach-related dynamics in our study, we were prompted to investigate whether other types of cell ensembles are also reactivated during sleep. To address this, we calculated the activation rates during both SWS and REM stages of rest 1 and rest 2 for each cell ensemble (see Methods).

Median activation rates across pre- and post-sleep stages were computed for each recording session. Statistical analysis revealed higher activation rates during post-REM than pre-REM as well as higher activation rates during post-SWS than pre-SWS, suggesting the potential significance of after-training sleep in memory processing (Table 3, Figure 4.A). In addition, the average activation rates during REM stages were significantly higher than during SWS stages (Table 3, Figure 4.A).

Reactivation strength is typically measured as the difference between the activity of online-related neural ensemble during pre-sleep and post-sleep. Here we aimed to examine whether reactivation strength differs between REM and SWS. However, the disparities in the activation rate scales between REM and SWS, both before and after sleep, we calculated the reactivation strength by quantifying substantial changes during post-sleep compared to pre-sleep for each stage, as follows:

$$Reactivation\ strength = \frac{activation\ rate_{post} - activation\ rate_{pre}}{activation\ rate_{pre}}$$

Our results showed that reactivation strength in SWS was significantly higher than REM. Importantly, this pattern was similar across all reach-related ensemble groups (Figure 4.B, Wilcoxon signed-rank test, C1: n=47, statistic = 116, $p=5.58 \times 10^{-7}$, C2: n=58, statistic= 98, $p=4.49 \times 10^{-9}$, C3: n=46, statistic = 105, $p=2.11 \times 10^{-7}$, C4: n=48, statistic = 177, $p=8.29 \times 10^{-6}$).

After analyzing sleep-reactivation for each reach-related cell ensemble groups, we explored whether overall ensembles' reactivation strength varied across training days regardless of their online dynamics. In this case, we compared the average

reactivation strength across four reach-related ensembles during recording sessions in the early phase (days 1-5, all animals) and the late phase (days 10-15, all animals except rat 2 which days 5-10 were considered). We determined reactivation changes for each sleep stage and with different combination of animals. Here is a summary of the results:

- SWS reactivation strength variations across days: considering all rats as a single group, we did not find significant changes across days (Figure 4.C(i), Wilcoxon signed-rank test, p=0.4304). While rapid learners exhibited higher reactivation strength during the initial learning phase in compared to the late phase (Figure 4.C(ii), Wilcoxon signed-rank test, p=0.0488), we did not find significant changes in the gradual learners (Figure 4.C(iii), Wilcoxon signed-rank test, p=0.1934).
- REM reactivation strength variations across days: considering all rats as a single group, we did not find significant changes across days (Figure 4.D(i), Wilcoxon signed-rank test, p=0.165). While rapid learners exhibited higher reactivation strength during the initial learning phase in compared to the late phase (Figure 4.D(ii), Wilcoxon signed-rank test, p=0.0371), we did not find significant changes in the gradual learners (Figure 4.D(iii), Wilcoxon signed-rank test, p=0.6953).

Together, our results suggest that skill-related cell ensembles with various activity profiles are reactivated during the post-raining sleep. Furthermore, reactivation was observed in both REM and SWS, with stronger strength during SWS, suggesting the potentially more contribution of this stage in the skill consolidation process.

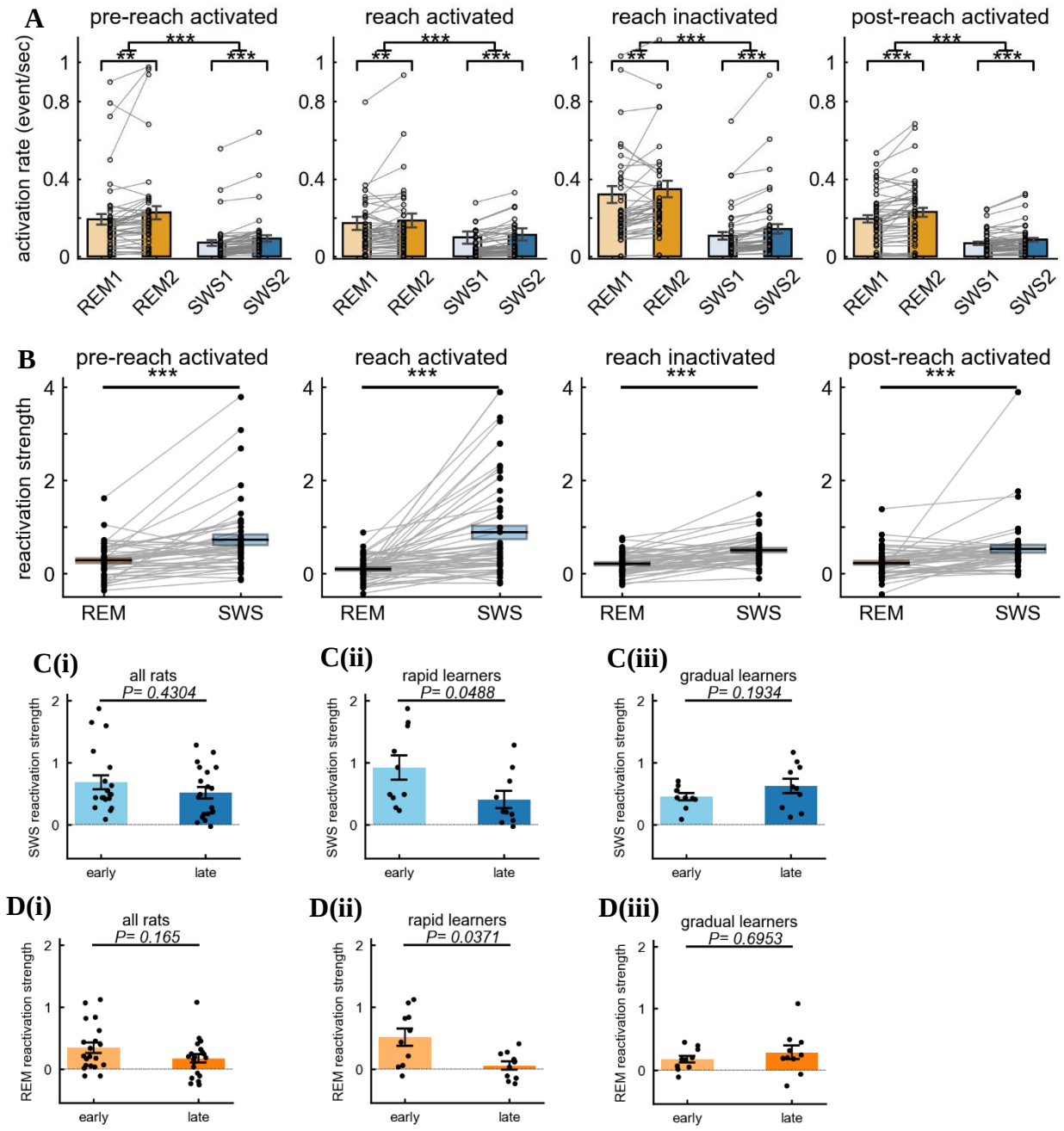


Figure 4. cell ensembles reactivation during sleep. A, activation rate during REM2 was significantly higher than REM1 for all reach-related groups (Wilcoxon signed-rank test: C1: n=47, p=6.66 $\times 10^{-4}$, C2: n=58, p=0.0055, C3: n=46, p=0.0073, C4: n=48, p=5.43 $\times 10^{-5}$, mean ± sem). Activation rate during SWS2 was significantly higher than SWS1 in all reach related groups (Wilcoxon signed-rank test: C1: n=47, p=7.99 $\times 10^{-8}$, C2: n=58, p=6.10 $\times 10^{-8}$, C3: n=46, p=1.56 $\times$ 10^{-12}, C4: n=48, p=1.22 $\times 10^{-8}$). In addition, average activation rate during REM was significantly higher than SWS (Wilcoxon signed-rank test: C1: n=47, p=1.71 $\times 10^{-8}$, C2: n=58, p=9.33 $\times 10^{-10}$, C3: n=46, p=3.97 $\times$ 10^{-13}, C4: n=48, p=1.47 $\times 10^{-12}$).

Figure 4. *continued*. B, Reactivation strength during REM and SWS in different reach-related ensemble groups (Wilcoxon signed-rank test; C1: n=47, $p=7.97 \times 10^{-7}$, C2: n=58, $p=2.81 \times 10^{-9}$, C3: n=46, $p=2.70 \times 10^{-7}$, C4: n=48, $p=8.29 \times 10^{-6}$, boxes show mean ± sem across sessions).

C, SWS overall reactivation strength during early days (days 1-5) versus late days (days 10-15, except rat 2: days 5-10); Wilcoxon signed-rank test, C(i): all rats; p=0.4304, C(ii): rapid learners; p=0.0488, C(iii): gradual learners; p=0.1934; mean ± sem across sessions.

D, REM overall reactivation strength during early days (days 1-5) versus late days (days 10-15, except rat 2: days 5-10); Wilcoxon signed-rank test, C(i): all rats; p=0.165, C(ii): rapid learners; p=0.0371, C(iii): gradual learners; p=0.6953; mean ± sem across sessions.

Table 3. Statistical results of activation rate comparison across different sleep stages

(Wilcoxon signed-rank test, Figure 3.A)

Reach group	REM1 vs REM2		SWS1 vs SWS2		REM vs SWS	
	statistic	*pvalue*	*statistic*	*Pvalue*	*statistic*	*Pvalue*
C1 (n=47)	216	6.66×10^{-4}	42	7.99×10^{-8}	18	1.71×10^{-8}
C2 (n=58)	440	0.0055	156	6.10×10^{-8}	65	9.33×10^{-10}
C3 (n=46)	298	0.0073	11	1.56×10^{-12}	6	3.97×10^{-13}
C4 (n=48)	211	5.43×10^{-5}	88	1.22×10^{-8}	17	1.47×10^{-12}

3.5. Higher reactivation during SO-coupled spindles

Building on our earlier findings of cell ensembles with different activity profiles during online training and their reactivation during sleep and as well as recognizing the potential significance of SWS in the reactivation, we conducted an investigation to determine if there were timing differences in reactivation during SWS among these various cell ensembles. Spindles are widely acknowledged as critical periods for the consolidation of procedural memory (Walker et al., 2002; Stickgold and Walker, 2007; Ramanathan et al., 2015; Boutin et al., 2018).

To determine the potential reactivation of cell ensembles during spindles, we first assessed whether the activation strength during spindles was higher in the post-SWS compared to the pre-SWS. Our results consistently indicated significant higher neural activity in the period of ± 1 sec around the spindles' center during post-spindles compared to the pre-spindles across all reach-related groups (Figure 5.A, Wilcoxon signed-rank test, C1: n=47, statistic=90, $p=2.92 \times 10^{-8}$, C2: n=58, statistic=249, $p=2.65 \times 10^{-6}$, C3: n=46, statistic=97, $p=1.07 \times 10^{-7}$, C4: n=48, statistic=89, $p=1.34 \times 10^{-8}$). Next, we further examined post-sleep spindles as potential candidates for reactivation during SWS and compared the average activation strength during spindle events with the average activity during non-spindle periods, which were determined from shuffled data (see Methods). Our results showed that cell ensembles of all clusters were more activated during spindle events. These findings emphasize the potential significance of spindle events as crucial periods during sleep for memory processing (Materials and Methods, Figure 5.B, Wilcoxon signed-rank test, C1: n=47, statistic=141, $p=1.61 \times 10^{-6}$, C2: n=58,

statistic=50, p=1.05 × 10^{-9}, C3: n=46, statistic=182, p=4.04 ×10^{-5}, C4: n=48, statistic=155, p=1.53 × 10^{-5}).

Expanding our research into the nuances of spindle-related memory consolidation, we explored the variations in activation strength between spindle events nested within the SOs (SO-coupled spindles were defined as the events within maximum ± 500 msec from the nearest SO trough) and the spindles observed away from the SOs (isolated spindles). Our analysis unveiled a significantly higher activation strength during the SO-coupled spindles compared to the isolated spindles in all reach-related groups (Figure 5.C, Wilcoxon signed-rank test, C1: statistic=95, p= 1.12 × 10^{-6}, C2: statistic =84.5, p= 5.86 × 10^{-8}, C3: statistic=105, p= 8.78 ×10^{-6}, C4: statistic= 134.5, p= 5.45 × 10^{-6}).

We also analyzed the activity profiles of cell ensembles around the spindles' onset. The analysis of peri-SO-coupled spindle time histogram revealed a brief and substantial suppression approximately 100 msec before the spindle onset, followed by a sharp increase in the activation starting from the spindle onset and maintained significantly higher than baseline (defined as the average activity between (-4, -2) sec and (2, 4) sec relative to the spindle onset) for about 700 msec (Figure 5.D, Wilcoxon signed-rank test, $p<0.001$, Bonferroni corrected). A similar analysis for isolated spindles showed a brief period of significantly increasing activation starting around 300 msec after the spindle onset. It was also observed a 800 msec period of suppression activity before the isolated spindle starting point (Figure 5.E, Wilcoxon signed-rank test, $p<0.001$, Bonferroni corrected). Importantly, we observed no significant differences in the temporal dynamics or the period of significantly higher

reactivation among different reach-related ensembles. This finding suggests that the initial periods of SO-coupled spindles are the preferred times for M1 cell ensembles' reactivation.

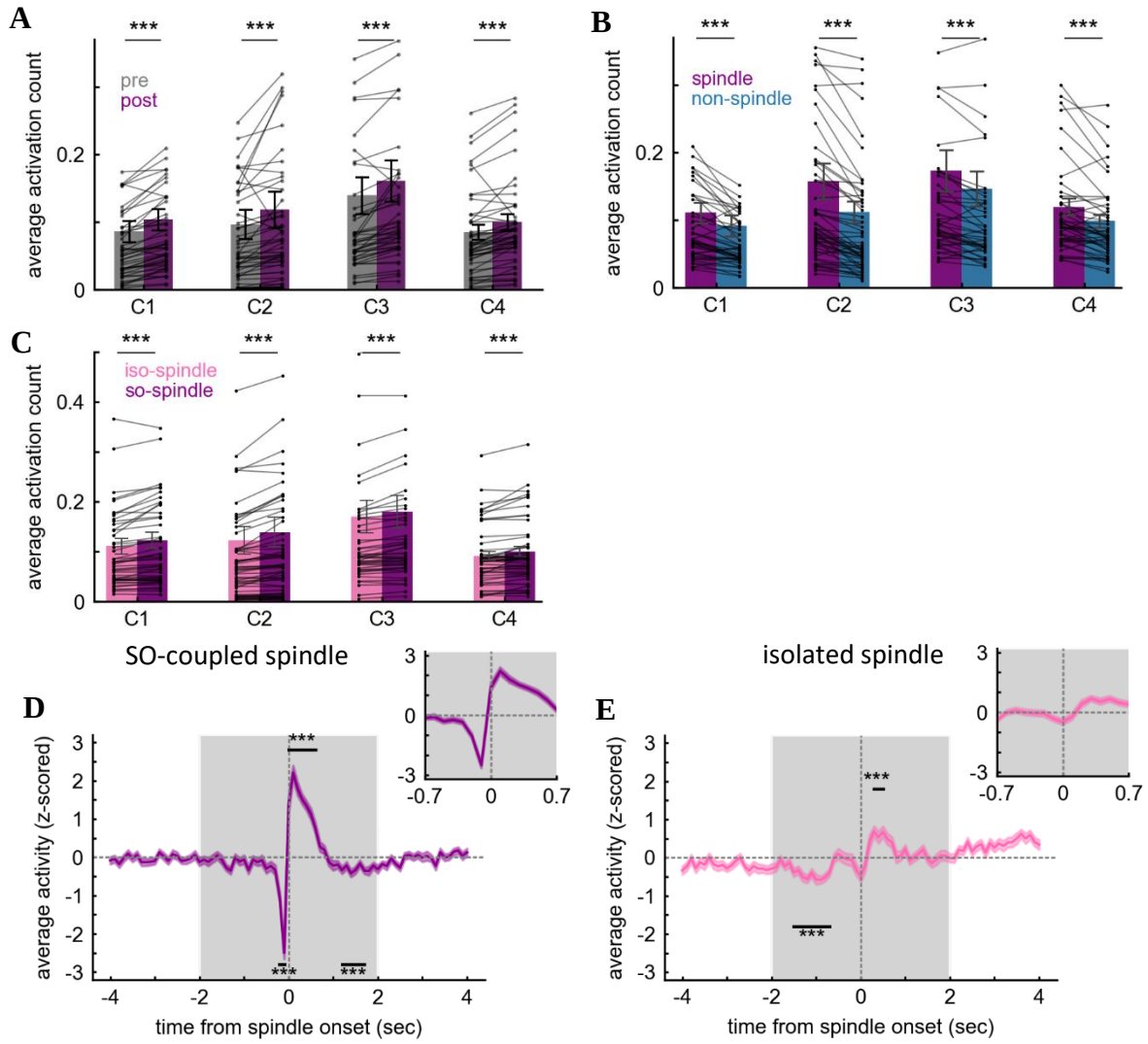


Figure 5. cell ensembles activity during spindles. A, comparison of ensembles' average activity during pre-sleep and post-sleep in reach related groups (mean ± sem, Wilcoxon signed-rank test, C1: n=47, statistic=90, p=2.92 × 10-8, C2: n=58, statistic=249, p=2.65 × 10^{-6}, C3: n=46, statistic=97, p=1.07 ×10^{-7}, C4: n=48, statistic=89, p=1.34 × 10^{-8}).

B, comparison of ensembles' average activity during post-sleep sleep spindles and random non-spindle shuffled events (mean ± sem, Wilcoxon signed-rank test, C1: n=47, statistic=141, p=1.61 × 10^{-6}, C2: n=58, statistic=50, p=1.05 × 10^{-9}, C3: n=46, statistic=182, p=4.04 ×10^{-5}, C4: n=48, statistic=155, p=1.53 × 10^{-5}).

Figure 5. *continued*. C, comparison of ensembles' average activity in isolated spindles versus SO-coupled spindles during post-sleep (Wilcoxon signed-rank test, C1: statistic=95, p= 1.12×10^{-6}, C2: statistic =84.5, p= 5.86×10^{-8}, C3: statistic=105, p= 8.78×10^{-6}, C4: statistic= 134.5, p= 5.45×10^{-6}). D, peri-event time histogram of ensembles activity around the SO-coupled spindle onset during post-sleep (n=60, mean ± sem across sessions). Horizonal black solid lines indicate the period that activation was significantly different than the baseline activation (defined as the average activation strength from (-4, -2) sec and from (2,4) sec relative to the spindle onset) (Wilcoxon singed-rank test, Bonferroni corrected, *** $p<0.001$). E, peri-event time histogram of ensembles' activity around the isolated spindle onset (n=60, mean ± sem across sessions). Horizonal black solid line indicates the period that activation was significantly different than the baseline activation (defined as the average activation strength from (-4, -2) sec and from (2,4) sec relative to the spindle onset) (Wilcoxon signed-rank test, Bonferroni corrected, *** $p<0.001$).

3.6. Suppression and Enhancement of M1 cell ensembles around hippocampal SWRs during SWS

Cortico-hippocampal interactions during non-REM have been proposed as a critical process for the formation of well-stabilized memories, particularly in the case of declarative memories (Marr, 1970; McClelland et al., 1995; Buzsáki, 1996; O'Neill et al., 2008; Sirota et al., 2008; Hahn et al., 2012). These dialogues are primarily coordinated with hippocampus SWRs (Sirota et al., 2003; Peyrache et al., 2009; Rothschild et al., 2017). However, in the context of non-declarative sensorimotor tasks, the extent to which the hippocampus activity during sleep is involved in the skill learning remains unclear. Here, our main goal was to explore how communication between the hippocampus, which plays a crucial role in encoding contextual information, and the primary motor cortex, a key area for motor skill learning, during sleep, relates to the learning process. To address this objective, we investigated how cell ensembles in the primary motor cortex modulate their activity during SWRs, comparing their activity during the ripple periods to that during randomly shuffled events during SWS. Our analysis revealed distinct patterns of

modulation in the ensembles surrounding SWRs during SWS including suppression, activation, and no-preference modulation (Figure 6.A). The SWR-suppressed group showed a plateau of inhibitory activity around the ripple periods, indicating a deliberate avoidance of activation during ripples, while SWR-activated cell ensembles exhibited increasing activity, with a peak occurring just before the center of the ripples (Figure 6.A). An analysis of the percentage of ensembles belonging to each of these three groups indicated that the majority of M1 cell ensembles (71.34% ±1.30%, mean ± sem across sessions) did not show a preferred modulation toward SWRs and there was no significant difference in the proportion of inhibition and enhancement groups across all recording sessions (Figure 6.B, Kruskal-Wallis, $p=7.84 \times 10^{-27}$, Dunn's post hoc test, Bonferroni correction). In detail, approximately 12.64% ±1.30% (mean ± sem across sessions) of reach-related M1 ensembles reactivated or replayed during hippocampal SWRs, while 16% ± 1.47% (mean ± sem across sessions) demonstrated avoidance of from activation during ripples.

We further conducted a more in-depth analysis to determine whether the level of suppression or activation was associated with progress of learning. In each recording session, we computed the Modulation Index, defined as the ratio of ensembles with increased modulation during SWRs to the total number exhibiting both increased and suppressed modulations. We subsequently compared the Modulation Index within different interactions of animals and training days using a 2x2 mixed design ANOVA, with considering the training phase (early: day 1-5 and late: day 10-15 in all rats, except rat 2: day 5-10) as a within-subject factor and the animal type (rapid and gradual) as a between-subject factor. We analyzed these

interactions separately for each reach-related cell ensembles groups.

Our results revealed that online activated cell ensembles with the pre-reach activated and reach-activated dynamics showed significant differences in modulation levels across learning days and between animals. Indeed, these cell ensembles in the rapid learners exhibited dominant suppression modulation during ripples in the early phase of training, followed by the enhancement activity in the late phase. In contrast, gradual learners displayed the opposite modulation pattern (2×2 mixed-design ANOVA, significant post-hoc t-test (Bonferroni corrected), pre-reach activated: Figure 6.C(i), interaction effect: $F(1,18)=9.8226$, $p=0.0057$, reach-activated: Figure 6.C(ii), $F(1,24)= 53.85$, $p=1.82 \times 10^{-7}$, ** $0.001<p<0.01$, ***$p<0.001$). In addition, our statistical analysis provided significant differences in SWR-reactivation modulation strength between rapid and gradual learners during phases of training. In pre-reach related ensembles, the amount of SWR-reactivation was significantly higher during the later phase of training in rapid learners compared to gradual learners (Figure 6.C(i)). In reach-activated ensembles, the level of SWR-reactivation was higher during the early days in rapid learners compared to gradual learners; however, this pattern was reversed during the late days (Figure 6.C(ii)). We did not find significant interactions in other reach-related cell ensemble groups (Figure 6.C(iii): reach-inactivated, $F(1,19)=1.5565$, $p=0.2273$, Figure 6.C(iv): post-reach activated, $F(1,19)=3.0393$, $p=0.0974$).

Together, these results suggest M1 cell ensembles exhibit diverse interactions with sleep-associated SWRs during different phases of motor learning which might be contributed to the skill improvement.

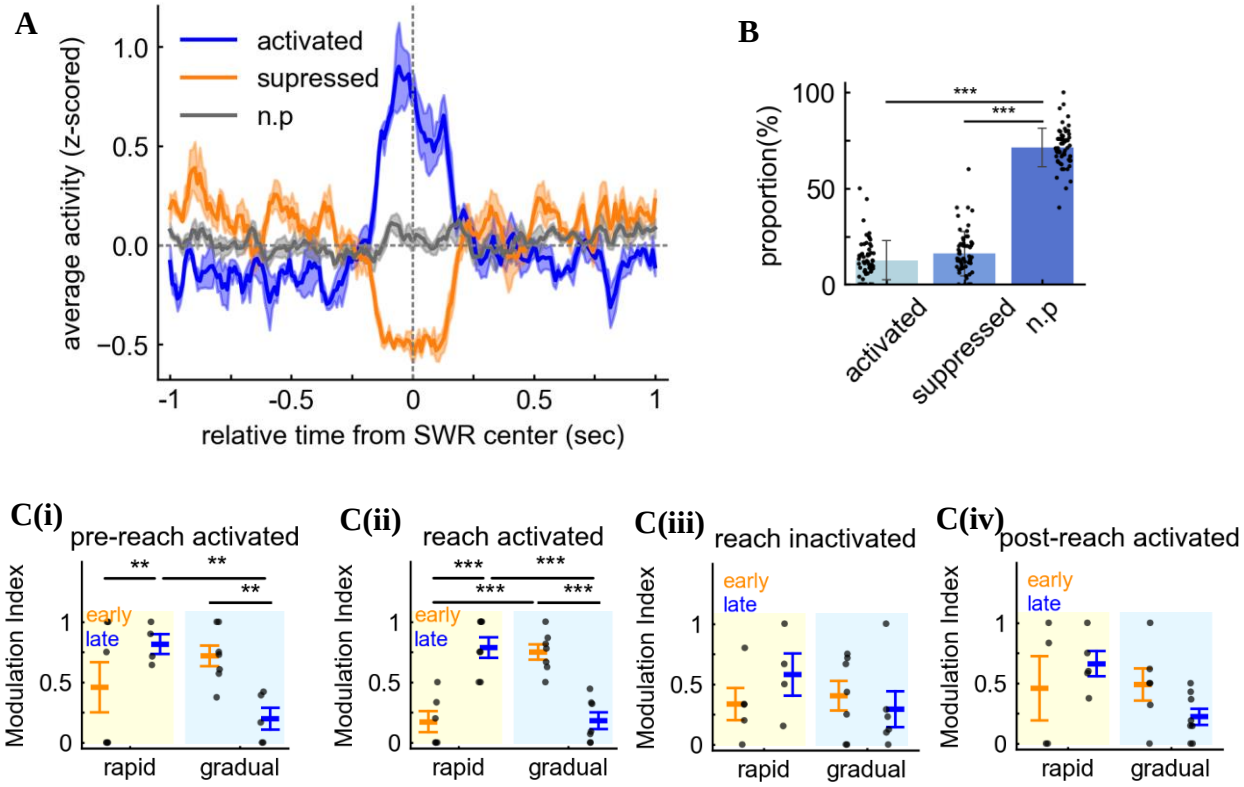


Figure 6. M1 Cell ensembles' modulation around sleep-SWRs. A, M1 reach-related cell ensembles exhibited various dynamics around SWRs (mean ± sem, blue: activated modulation, orange: suppressed modulation, gray: no preference modulation (n.p). B, percentage of different SWR modulation types across different recording sessions (n=60, Kruskal-Wallis, $p=7.84 \times 10^{-27}$, Dunn's post hoc test with Bonferroni correction. C(i), Modulation Index for pre-reach activated cell ensembles (mean ± sem, 2-way mixed design ANOVA ($F(1,18)=9.8226$, $p=0.0057$, significant post-hoc t-test (Bonferroni corrected), ** $0.001<p<0.01$). C(ii), Modulation index for reach activated cell ensembles (mean ± sem, 2-way mixed ANOVA, interaction effect, $F(1,24)= 53.85$, $p=1.82 \times 10^{-7}$, significant post-hoc t-test (Bonferroni corrected), ***$p<0.001$). C(iii), Modulation Index for reach inactivated cell ensembles (mean ± sem, $F(1,19)=1.5565$, $p=0.2273$). C(iv), Modulation Index for post- reach activated cell ensembles (mean ± sem, 2-way mixed ANOVA, $F(1,19)=3.0393$, $p=0.0974$).

3.7.Temporal relationship between SWRs and SO-coupled spindles

As our results indicated varying levels of SWR-modulation across learning days and among rat types, we further explored the degree of temporal coupling between SO-spindles onset, shown as a preferred timing of M1 cell ensembles reactivation during SWS, and SWR center. Each SWR in the rest 2 was linked to the nearest SO-spindle and then peri-event time histogram of SWR occurrences around (-2,2) sec from the onset of SO-spindle was created (see Methods). The average PETH was created within different combination of animals including all rats as a single group (Figure 7.A(i)), rapid learners (Figure 7.A(ii)) and gradual learners (Figure 7.A(iii)).

We then examined the variations in the degree of temporal coupling between SWR and SO-spindles across learning days. As the main objective was to find the changes in the amount of coupling regardless of the direction of coupling, we obtained the absolute value from the PETHs and identified the corresponding time at their peak amplitude. Initially, we pooled all rats as a single group and compared the lag values across the recording sessions of early and late phases of training. The findings showed no statistical significance (Figure 7.B(i), Wilcoxon signed-rank test, p=0.6477). We further explored interactions between temporal relationship of these two events during the early and late phases of training across different animal types. Our results revealed that rapid learners exhibited a significantly lower degree of temporal nesting during the late phase compared to the early phase (Figure 7.B(ii), Wilcoxon signed-rank test, p=0.0273). Conversely, gradual learners showed a significantly higher degree of temporal coupling during the later days compared to the earlier days (Figure 7.B(iii), Wilcoxon signed-rank test, p=0.0371). In summary,

these results suggest that the amount of temporal nesting of SWRs and SO-spindles undergoes changes during training days, and it may be correlated with the varying levels of interactions between the motor cortex and the hippocampus.

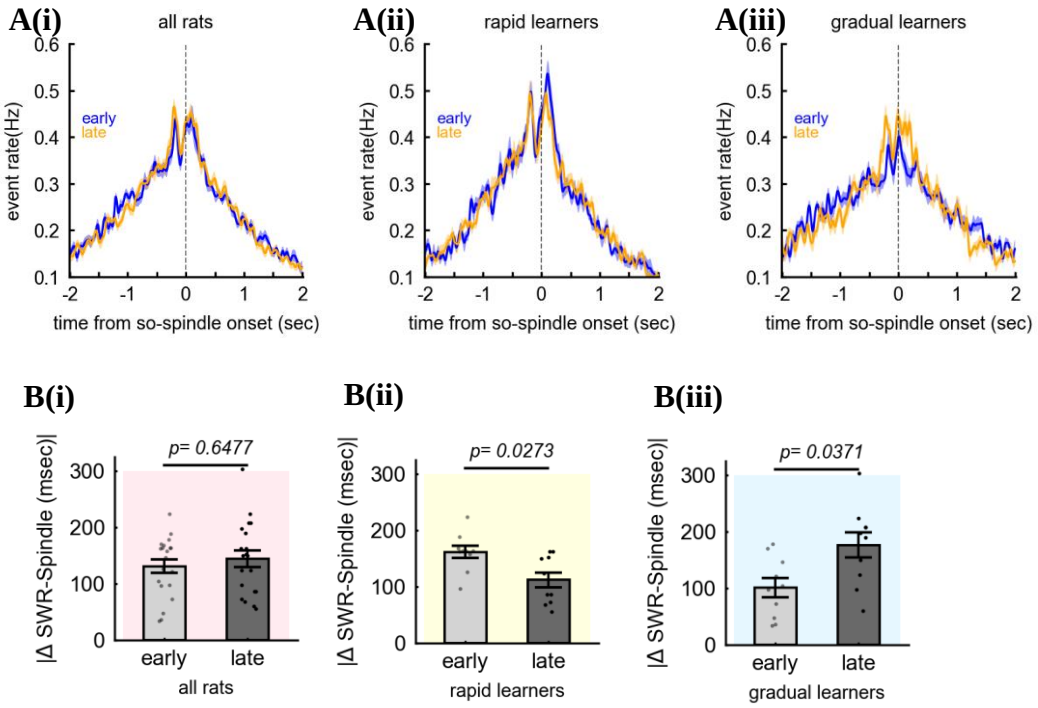


Figure 7. Temporal relationship between SWRs and SO-spindles. A, peri-event time histogram of ripple occurrences around the onset of so-spindles during early and late days of training (mean ± sem across sessions, blue: early phase, orange: late phase. A(i): all rats, A(ii): only rapid learner, A(iii): gradual learners. The y-axis shows the event rate in Hertz (raw histogram values were normalized by the (number of bins × temporal resolution).

B, absolute temporal lag between SWR and SO-spindles at the maximum portion of events coupling during early and late days of training, Wilcoxon singed-rank test, B(i): all rats (n=40 sessions, statistic= 92.5, p=0.6477), B(ii): rapid learners (n=10 sessions, statistic= 6, p=0.0273), B(iii): gradual learners (n=10 sessions, statistic= 7, p=0.0371). mean ± sem across sessions.

4. Discussion

Motor skill learning involves complex neural patterns due to the high-dimensional parameter space required for executing precise movements. While extensive research has explored motor learning mechanisms during both online and offline processing phases, much of the focus has been confined to specific aspects of the learning process. Our investigation aimed to bridge this gap by exploring the correlations between various cell ensembles' dynamics in the rat's primary motor cortex during both online and offline learning periods. We used an unsupervised framework to analyze neural dynamics related to skilled reaching tasks during online training and explored the role of sleep reactivation in skill enhancement, focusing on inter-region processing and M1 interactions with the hippocampus. Our data analysis revealed various dynamics in ensembles' activity during task execution, and these ensembles were subsequently reactivated during post-training sleep, specifically during SO-coupled spindles. Furthermore, we identified distinct activation patterns of M1 ensembles during SWS-ripples, which may potentially contribute to different strategies associated with the integration of contextual and skill-related information in the sensorimotor tasks. In addition, temporal coupling between SO-spindles and SWRs may coordinate these different modulations throughout different levels of skill consolidation.

4.1. Unsupervised framework for analysis of neural activity during online and offline periods

In this study, we employed an unsupervised framework for sleep-associated

memory study. Regarding the main advantage of this approach, it provided us with an unbiased scaffold for identifying candidate cell ensembles during the recording sessions. We ran CAD through the entire recording and extracted significant cell ensembles activated without taking account to a specific course of recording either behavior period or sleep. Indeed, memory analysis was performed following the cell ensemble detection and without any prior assumptions about the behavioral state. Moreover, previous analyses of sleep associated with skill learning mostly used dimensionality reduction methods, specifically PCA, which had their own limitations including linearity, orthogonality, sensitivity to local non-stationarities, and as well providing limited number of synchronous neural activity patterns (Gulati et al., 2014; Ramanathan et al., 2015; Gulati et al., 2017; Kim et al., 2019; Eckert et al., 2020). Here we detected more neural activity patterns within different correlation orders and population sizes, which allowed us to contribute a comprehensive study of neural information processing in the motor learning tasks. In summary, our proposed framework suggested a powerful and impartial analysis of neural data associated with memory and learning.

4.2. Properties of cell ensembles in the primary motor cortex

In our exploration on the structure of neural firing patterns within M1, we observed that the major cell ensembles exhibited synchronous or relatively short sequential activations, suggesting the precise activation structure of cell ensembles (Figure 2.E). These findings are consistent with the notion of precise coordination across neural activities associated with motor learning, reported in previous studies

of motor cortex neural activities (Riehle et al., 1997; Torre et al., 2016). Although these studies focused on detecting synchronous patterns, here we did not assume this constraint in our analysis and revealed dominant synchronous activation of cell ensembles in the primary motor cortex. An explanation for the synchronization of neural activities in M1 might be rooted in the inherently complex and diverse nature of motor tasks. These experiences are impacted by large variations in several factors such as task goals, kinematics complexity, initial condition, biomechanics information, feedback and sensory inputs, muscle fatigue, and environmental noise. Considering the rapid nature of motor actions, the cortical network should engage in precise computations to optimize and control movements across a spectrum of scenarios. The precise synchronization of neural activity may serve as a fundamental mechanism for ensuring that the motor cortex can adapt to these diverse demands efficiently.

Moreover, the relative size of the detected cell ensembles raises a question of how our cell ensemble detection involved with detecting large units. Our results indicated relatively small ensemble sizes in the smaller bins (2-3 neurons) and slightly larger population sizes (4-6 neurons) in the larger temporal resolutions (Figure 2.D). These findings invite various interpretations from different perspectives, considering both computational methodologies and neural mechanisms. Firstly, the CAD method incorporates strict statistical significance levels by using Bonferroni correction at each agglomerative step, which can lead to the dominance of relatively small-sized ensembles. Our results might be compared with subspace neural data analysis methods such as PCA or ICA which have been widely used in

the study of neural activity in both episodic and implicit learning tasks. While these methods typically capture population-level effects, it is noteworthy that a small subset of neurons (comprising 2-3 neurons) exhibited significantly higher weights and, consequently, made a substantial contribution to the resulting principal components or independent components (Van de Ven et al., 2016; Kim et al., 2019). These observations suggest that those selected neurons play dominant roles in shaping neural activities associated with the behavior state, and can be viewed as significant cell assembly units. It should be noted that due to the lack of ground truth, different cell assembly computational methods may provide different results in terms of size, structure and member neurons (Watanabe et al., 2019). Secondly, from a neural processing viewpoint, the presence of small-sized cell ensemble units may reflect a finely tuned specialization for distinct motor functions. This fine-tuning likely structure underlies the exceptional precision observed in the execution of fine motor actions. In addition, such ensemble structures might be more efficient in detecting and correcting errors during motor execution, enhancing the overall robustness of motor system and promoting error-free communication while maintaining network stability and conserving more energy to optimize computational resources associated with the complex and diverse motor movements. As we suggested, the activation of different cell ensembles in a sequential order during the reaching task, also supports the hypothesis of chunking-based information processing in the brain (Wickelgren, 1992). It should be emphasized that optimal size of cell ensembles remains a conundrum due to the involvement of various factors, specifically the main goal of ensemble's activation from the underlying reader neurons (Buzsáki, 2010).

Simulation studies of motor cortex neural activities will be helpful to understand the ensembles structures.

In summary, our findings highlight the intricate yet efficient organization of neural ensembles, where discrete cell ensembles with small and short-term firing configurations potentially may be involved in shaping motor behaviors, maintaining precision, conserving resources, and facilitating adaptability.

4.3. Neural activity dynamics during online learning

After detecting of multiple cell ensembles, we conducted clustering analysis on the ensemble PETHs in the window of ± 5sec centered around the object touching time. Our results revealed four distinct types of neural dynamics during reach-to-grasp task, each with unique temporal profiles and potential functional implications (Figure 3.A). These dynamics encompassed preparatory activities, enhancements before and after grasping, and suppression modulation during execution of motor action. These variabilities the in neural dynamics are in align with previous research the complexity of neural activities associated with motor learning tasks (Rokni et al., 2007; Peters et al., 2014; Liberti III et al., 2016). A study of mice neural activity reported three main dynamics of cortico-spinal neurons associated with a lever-press task including movement-activation, movement-suppression and non-discriminatory activation (Peters et al., 2017). While, previous PC analysis of neural ensembles during the skilled-reaching task identified the neural dynamics similar to the reach-activated ensembles (C2) in our study (Eckert et al., 2020), here we uncovered additional neural dynamics involved in this task. Moreover, considering their relative

dynamics around the reaching task, it can be inferred that the single-pellet reaching task is accomplished through a sequential activation of different cell ensembles. This expansion of extracted dynamics highlights the richness and complexity of neural processing within the motor cortex during the action execution.

We also observed the consistent observation of those four dynamics across learning days and animals (Figure 3.B-F), suggesting their fundamental role in the neural processes underlying the reaching task. Indeed, our study has also raised the intriguing possibility that changes in the coordination of those dynamics may be involved within the skill enhancement over the course of training days.

Together, these insights contribute to the broader discourse on neural plasticity and motor learning, further advancing our understanding of the intricate neural mechanisms associated with motor learning.

4.4. Reactivation of reach-related cell ensembles during offline periods

After identifying ensembles involved with the reaching task, we proceed to analyze their activity during sleep. We quantified cell ensembles' frequency of activation during REM and SWS stages in both pre- and post-sleep periods. While previous studies indicated the reactivation of online neural ensemble-with similar dynamic to the reach-activated group (C2) in our study- during the sleep (Ramanathan et al., 2015; Eckert et al., 2020), we conducted sleep-reactivation analysis for cell ensembles of each reach-related group independently to determine whether there were any differences in their activity during offline periods.

Our findings uncovered higher activation of all reach-related cell ensembles

during the post-sleep in compared to the pre-sleep, suggesting the potential reactivation of task-related neural ensembles after experience of skill training. During the second rest period, the brain appears to intensify its efforts in processing and consolidating memories acquired during preceding wakefulness. One intriguing aspect of our findings involves cell ensembles that exhibited suppression activity during the task but displayed activation during sleep periods. Typically, standard memory reactivation involves the reactivation or replay of task-emergent ensembles during the sleep. However, these specific ensembles were inactive during movement. While inhibitory spatiotemporal patterns of neural activity have been proposed to be essential for stabilizing the motor network (Liberti III et al., 2016), it's plausible that their activation during sleep is also associated with the network stabilization process as a part of memory reorganization during sleep. We also analyzed activation tendency during sleep from two aspects:

1. Activation tendency during pre-sleep (pre-activation) versus post-sleep (reactivation): We compared cell ensembles' activation rate for each sleep stage between corresponding sleep periods. We observed significantly higher activation rate during the post-SWS in compared to the pre-SWS across all task-related cell ensemble groups (Figure 4.A). These findings align with the prior PC-reactivation analyses of M1 neural ensembles, which suggested heightened reactivation strength following motor learning experiences during SWS (Gulati et al., 2014; Ramanathan et al., 2015; Eckert et al., 2020). Additionally, We noted an increased activation rate of cell ensembles during post-REM in compared to pre-REM, with this trend consistent across all ensemble groups (Figure 4.A). A

recent study demonstrated the importance of REM sleep following rotarod motor learning in orchestrating modifications of postsynaptic dendritic spines within the mouse primary motor cortex and these dendritic spine changes were recognized as a crucial process contributing to the stabilization of spine networks, a vital component of synaptic consolidation (Li et al., 2017). In addition, human studies of perceptual memory as another type of procedural memory highlighted the dependency of memory consolidation on REM sleep (Karni et al., 1994). Moreover, studies focusing on declarative memories humans and animals have proposed the replay or reactivation of neural ensembles during REM prior experiencing (Louie and Wilson, 2001) or after training a behavioral task (Sterpenich et al., 2014). Although, a PC-reactivation analysis using the same dataset as our study emphasized the importance of REM sleep in the skill enhancement, it reported the higher reactivation strength during pre-REM (Eckert et al., 2020). One possibility for this discrepancy may stem from differences in reactivation analysis methodologies. In our approach, we directly examined neural data by extracting cell ensembles and quantifying their frequency of activation as a metric for reactivation measurement, whereas PC-reactivation analysis determines the cumulative effect of all neural population activity weights in a lower dimensional space than the original neural space. It's crucial to acknowledge that without a definitive ground truth, different computational methods might lead to variations in neural ensemble detection and reactivation measurements.

2. Reactivation tendency between sleep stages: Our analysis on the relative changes

of activation from the pre-sleep to the post-sleep in different sleep stages showed significant higher reactivation changes during the SWS compared to the REM sleep, (Figure 4.B) hinting at the potentially leading role of SWS in fine-tuning and stabilizing motor memories associated with the reach-to-grasp task, with a complementary role of REM for offline memory processing. These findings are consistent with previous study of human target-memory reactivation suggested the involvement of both REM and SWS in the memory consolidation after experience of motor sequence learning task (Cousins et al., 2016).

In short, our results suggest the potential contribution of both REM and SWS stages in the consolidation of motor memories in distinct, yet complementary ways and their temporal coordination may play different roles in the skill refinement process.

4.5. Offline reactivation changes across training days

So far we investigated the cell ensembles' reactivation strength for each derived task-related groups, independently. As we also interested in analyzing whether reactivation strength during REM or SWS may vary over the course of learning, we computed the averaged reactivation strength across task-related ensembles groups for each recording session. The outcome from rapid learners indicated significant higher reactivation strength in both REM and SWS during the initial phase of training (Figure 4.C(ii), D(ii)), although we did not find significant variations in neither REM nor SWS when considering gradual learners (Figure 4.C(iii), D(iii)). The results obtained from rapid learners support prior research demonstrating heightened

reactivation strength during the initial stages of skill learning (Ramanathan et al., 2015; Eckert et al., 2020). Notably, our study revealed a divergence in the outcomes observed in gradual learners, where we did not identify significant changes in reactivation between the early and late phases of learning. These discrepancies suggest that sleep might serve dual functions in memory processing based on the initial level of skill acquisition: (a) facilitating the swift acquisition of skills by generating strong reactivation strengths during the initial phase or (b) strengthening skill consolidation towards more advanced levels by maintaining a specified range of reactivations across subsequent learning days.

4.6. M1 ensembles reactivation during SWS coincides with SO-coupled spindles

We next delved into the reactivation dynamics during SWS, considering the hypothesized role of spindles in memory consolidation, as highlighted in numerous human and animal studies (Nishida and Walker, 2007; Peyrache et al., 2009; Johnson et al., 2010; Barakat et al., 2011; Mednick et al., 2013; Lustenberger et al., 2016; Boutin and Doyon, 2020). Additionally, it has been suggested that the interplay between fast-spindles and SOs may facilitate memory consolidation and synaptic plasticity (Niethard et al., 2018; Kam et al., 2019; Silversmith et al., 2020).

Given that we had classified cell ensembles based on their temporal activity profiles during online training, we initially aimed to investigate whether there were differences in the timing of their reactivation during SWS. Considering spindles as significant candidates for neural ensemble reactivation, our examination of the timing of potential reactivation within each reach-related ensemble group yielded consistent

results: all groups displayed significantly higher reactivation during fast-spindle events coupled with SOs in SWS (Figure 5.B,C). Indeed, we initially verified the significantly stronger activation during fast-spindles in compared to the inter-spindle intervals. Then, we found significantly stronger activation during spindle events that were more closely aligned with the SOs (referred to as SO-coupled spindles) compared to those observed with a larger delay from the nearest SO (referred to as isolated spindles). It's worth noting that fast-spindles are typically generated during the SO up-state (Johnson et al., 2010), but they have been shown to be activated with various levels of delay from SOs (Silversmith et al., 2020). Pyramidal neural activity during precise nesting events (SO-coupled spindles) were found to be higher than in cases of large delays (isolated spindles) (Niethard et al., 2018). Therefore, while both SOs and spindles are transitory events leading to changes in neural activity states, the level of nesting between spindles and SOs can distinguished spindles as two different events that may influence reactivation tendencies. In addition, a recent optogenetic study of BMI[1] task indicated the presence of different roles for fast-spindles linked with SOs or delta waves in memory processing related to skill learning (Kim et al., 2019). It's possible that isolated spindles in our study, which occurred outside of the defined window for coupling, were associated with delta waves.

Moreover, our exploration of the dynamics of ensemble's activation during SO-coupled spindles and isolated spindles revealed notable differences between these events. Cell ensembles exhibited a significantly high level of activation coinciding with the initiation of SO-coupled spindles onset, which was sustained for

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approximately 700 msec. A brief suppression of activation was observed, potentially rooted in the down-state phase of the SO when neural activity is temporarily silenced (Johnson et al., 2010) (Figure 5.D). However, isolated spindles were associated with a notably shorter period of significantly higher activation of cell ensembles compared to the baseline, and peak activation was relatively lower than during SO-coupled spindle (Figure 5.E).

Taken together, our findings support the notion that spindles coupled with slow oscillations are correlated with the reactivation of task-related cell ensembles regardless of their online dynamics, potentially facilitating the coordination and integration of these ensembles for the memory consolidation.

4.7. Dynamic interactions of motor cortex ensemble's activity during sleep SWRs

Large body of studies have reported the importance of cortico-hippocampal dialogue in creating a semantic and long-term declarative memory (Buzsáki, 1989, 1996; Sutherland and McNaughton, 2000; Battaglia et al., 2004; Peyrache et al., 2009), however the extent to which sensorimotor learning relies on hippocampal information has been a subject of debate in the field. While some literature has suggested the dissociation between procedural learning and the hippocampus (Squire and Zola, 1996; Gould et al., 2002), others, particularly those rooted in the human brain research, have highlighted the hippocampal involvement in skill learning (Albouy et al., 2008; Albouy et al., 2013; Scholz et al., 2015; Boutin et al., 2018; Burman, 2019). Our results contributed to this ongoing debate by identifying diverse communication patterns within the primary motor cortex cell ensembles during

hippocampus SWRs in SWS. These modulations included enhancement, suppression, and no preference modulation (Figure 6.A). These modulation diversities have been reported in the prefrontal cortex ensembles associated with the episodic memory (Tang et al., 2017). Given the non-declarative nature of motor learning tasks, which are not directly dependent on the hippocampus, it was expected that most of the detected cell ensembles would not exhibit a clear preference modulation in relation to hippocampal SWRs. Instead, we observed two other groups: inhibition and activation, distributed nearly equally across the detected ensembles (Figure 6.B). Cell ensembles with increasing activity during SWRs indeed indicate their potential reactivation during these events. A recent study of M1 neural ensembles activity during SWRs reported the reactivation of task-emergent neural population during post-sleep SWR suggesting the significance of the dialogue between the motor cortex and the hippocampus for skill consolidation (Kim et al., 2023). The discovery of suppression modulation introduces a potentially novel aspect, indicating the avoidance of M1 task-related cell ensembles' reactivation during sleep ripples. This inhibitory modulation has been proposed as a mechanism enabling the selective allocation, filtration, alternation, and transformation of specific memory traces, particularly in the declarative tasks (Jadhav et al., 2016; Noguchi et al., 2022). However, an alternative possibility, worthy of exploration in non-declarative tasks linked to contextual information, might involve the stage of hippocampal involvement in the skill consolidation process.

To better understand the implications of these varied modulations, particularly their potential effects on the learning performance, we conducted an analysis by

measuring the density of suppression and enhancement. Remarkably, our results revealed the dynamic interplay between the prevalent modes of M1 ensembles modulation in relation to the SWRs within different animal types and learning phases, suggesting two potential strategies for integrating contextual knowledge and skill information (Figure 6.C). The first strategy, observed in the rapid learners, involved initially prioritizing the acquisition of motor skills without immediate integration of contextual information. Once a certain level of skill proficiency was achieved, there was an apparent shift towards communication with the hippocampus to possibly integrate episodic information, ultimately enhancing skill performance. In contrast, the second strategy, exhibited by gradual learners, entailed continuous communication with the hippocampus from the onset of the learning process. However, as skill proficiency increased, there was a reduction in this communication, allowing for skill information to be processed independently, without integration of hippocampus information. Both strategies may have their merits in skill promotion. Although it appears that the first strategy, characterized by delayed contextual integration, could possibly be advantageous in fast motor execution. This suggests that the initial focus on skill acquisition followed by contextual memory integration may allow for efficient skill execution. On the other hand, the second strategy, which entails processing both contextual and skill information from the early training days followed by exclusive focus on skill information processing may confer advantages in terms of a more holistic understanding of the skill from the beginning, potentially contributing to improved long-term retention and adaptability.

The involvement of the hippocampus in the exploration phase (skill acquisition

phase) of motor skill learning has been proposed in a recent study of rats' M1 neural ensembles that learned the reaching task (Kim et al., 2023). Previous human fMRI studies have demonstrated the significant contribution of the hippocampus in different phases of online motor sequence learning. These include the initial motor acquisition phase (Orban et al., 2010) or both the early learning and consolidation phases (Gheysen et al., 2010). Our results extend this understanding by suggesting that hippocampal involvement and its role in memory processing could also continue during the sleep period after experiencing motor learning. In addition, the dynamical cortico-hippocampal communications during different phases of learning may contribute to different aspects of skill learning. In summary, our results argue that M1-hippocampal communication during sleep may follow dynamic patterns which selecting a specific strategy may correlate with the initial level of skill acquisition.

Furthermore, we also observed significant interactions of SWR-modulation in cell ensembles with the preparatory and increasing activity during the motor execution period (Figure 6.C(i, ii)) while no significant interactions were detected in other reach-related cell ensembles. One possible explanation for this finding can be attributed to the significance of contextual information, which is particularly relevant to the elements of the motor task that rely on it to enhance motor planning and action prediction. In the context of the skilled reaching task, this knowledge is likely correlated with the pre-reach and reach-activated cell ensembles, which play a leading role from the initiation of the reach to the grasping of the food. Contextual information inputs in motor computations can contribute to enhance the precision of the action (Wright and Shea, 1991). This might be included details such as spatial

information (e.g., cage physical dimensions, opening location, and food placement), visual cues (time when the food is placed), and olfactory cues in the rat's skilled reaching task, which are potential examples of sensory information that may be essential for the motor commands' computation. A previous human study indicated that sleep-consolidation enhances performance in tasks that involve implicit learning with contextual information. However, it does not significantly impact tasks that rely solely on implicit learning (Spencer et al., 2006). Given the connection between our results and these previous findings, we can infer that the initial phase of the reaching task (from paw advancing to grasping) is potentially tied to contextual information, while the subsequent withdraw movement can be seen as a more implicit or gross motor action.

In essence, our findings shed light the potential adaptability of mechanisms for dialogue between declarative and non-declarative memories during the sleep to suit different learning strategies according to the level of skill acquisition.

4.8. Variations of SWRs and SO-spindles' temporal coupling across training days

Considering various M1 modulations during SWRs across different days and animals, we explored whether the temporal alignment of SWR events with SO-coupled spindle events corresponded to these variations. While we did not observe a significant difference in the shifting of M1 ensembles' reactivation time from the onset of SO-spindles across learning days, it appears that relative shifts in the timing of SWRs in relation to SO-spindles contributed to the variations in M1 ensembles' activation patterns during ripples. Notably, in the case of rapid learners, SWRs tended

to occur more closely to SO-spindles on later days, resulting in ensemble's increased activation modulation during SWRs, compared to earlier days when they occurred farther from the spindles, leading to inhibitory modulation (Figure 7.B(ii)). Conversely, gradual learners exhibited the opposite trend (Figure 7.B(iii)). These observations suggest that dynamic interactions between SO-spindles and SWRs might result in diverse interactions between the motor cortex and the hippocampus, facilitating the integration of episodic knowledge and skill information in sensorimotor learning tasks. The significance of precise sequential coupling between spindles, SOs and SWRs for the hippocampal-cortical interactions associated with the memory consolidation has been proposed in both human and animal studies (Maingret et al., 2016; Staresina et al., 2023). An increase in the density of coupled SWR and SO events following the task, particularly in the initial phase of learning, has been reported in a recent study on rat's motor skill learning (Kim et al., 2023). In alignment with those findings, our results also emphasize that the degree of fine-temporal coupling between SO-spindles and SWRs might orchestrate different communication levels between the motor cortex and the hippocampus.

Furthermore, neocortical-hippocampal two-stage reciprocal communications have been suggested to facilitate the consolidation of declarative tasks (Marr, 1970; Buzsáki, 1989; McClelland et al., 1995; Buzsáki, 1996). While this model posits the potential variability of cortico-hippocampal communications during sleep across different phases of learning, the possibility of changes in the direction of dialogue across the long-term training is still a question. Although, the motor learning task is not directly rely on the hippocampus, our results did not show significant differences

in the coupling order of SWRs and so-spindles (Figure 7.A). Indeed, we found almost equal contributions of SWR occurrences before and after the SO-spindle onset in both early and late phases of learning, regardless of animals' online learning performance. As indicated, the cortico-hippocampal communication directionality is still undergoing research, which is beyond the scope of this research study. Here, we primarily interested in determining the mechanisms underlying the different dynamics of M1 ensembles during SWRs and demonstrated that changes in the degree of temporal nesting between ripples and SO-spindles may potentially contribute to these modulations.

5. Limitations and future work

While our current study has unveiled variable neural activity patterns during movement execution, the path forward in our research journey involves delving deeper into the intricate relationship between these neural patterns and the specific components of movement. To attain a more comprehensive understanding of how evolving neural activity orchestrates the generation of movement, we propose a promising avenue for future research: a holistic analysis that seamlessly integrates behavioral and neural data. Such an integrated approach can bridge the gap between observed neural dynamics and the actual execution of motor tasks, offering profound insights into the neural underpinnings of skilled movements.

Moreover, while our current investigation focused on analyzing the communication between the cortex and hippocampus during sleep-associated SWRs we recognize the potential value in exploring the distinctions between SWRs during wakefulness and those during sleep in the context of skill learning tasks. This further exploration could shed light on the nuances of the cortico-hippocampal dialogue in procedural learning, providing a more comprehensive understanding of how these interactions evolve across different phases of learning.

In addition, our study has highlighted the importance of reactivating M1 neural ensembles when spindles are nested within SOs, as well as the diverse modulation of these ensembles during hippocampal SWRs. Recent human studies have emphasized the significance of the coupling between SOs, spindles, and SWRs on neural representation processing (Staresina et al., 2023). Consequently, it would be worthwhile to investigate the impact of the coordination between hippocampal SWRs

and SO-coupled spindles with neural ensembles activity, aiming to uncover the potential effects of such coupling on long-term memory processing.

6. Conclusion

In summary, our study provides a comprehensive examination of neural ensemble dynamics in motor skill acquisition tasks during online and offline periods. We have uncovered intricate patterns of neural activity within the primary motor cortex, illustrating the brain's adaptability, precision in motor control, and memory consolidation mechanisms during sleep. Our unsupervised framework for neural ensemble detection also offers a powerful and unbiased tool for analyzing neural data associated with memory and learning.

During online learning, we identified distinct neural dynamics associated with reach-to-grasp tasks, revealing the complexity of neural processing during motor actions. Reactivation analysis during offline periods highlighted the importance of sleep as a temporal window for skill refinement and consolidation, with contributions from both REM and SWS sleep stages. Additionally, coincidence of spindles and SOs suggested the time for integration of online neural dynamics during SWS. Furthermore, our findings contribute to the ongoing debate regarding the role of the hippocampus in motor learning. Diverse communication modulations within primary motor cortex cell ensembles during hippocampal SWRs in SWS suggest two potential strategies for integrating contextual information and skill information during learning phases. In conclusion, our study advanced our understanding of the complex interplay between neural dynamics and motor learning during online and offline processing.

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